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Overexpression of glucosylceramide synthase in associated with multidrug resistance of leukemia cells

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Abstract

Ceramide, as a second messenger, initiates one of the major signal transduction pathways in tumor apoptosis. Glucosylceramide synthase (GCS) catalyzes glycosylation of ceramide and produces glucosylceramide. Through GCS, ceramide glycosylation allows cellular escape from ceramide-induced programmed cell death. Here we investigated the expression of GCS in human leukemia cells and an association between GCS and multidrug resistance of leukemia cells. Using RT-PCR technique the level of GCS gene was detected in 65 clinical multidrug resistance/non-resistance cases with leukemia, and in K562 and K562/A02 cell lines. AlamarBlue Assay was applied to confirm the multidrug resistant of K562/A02 cells. PPMP, which is a chemical inhibitor for GCS, was used to determine the relationship between GCS and drug-resistance in K562/A02 cells. In addition, multidrug resistance gene (mdr1), Bcl-2 and Bax mRNA was also analyzed by RT-PCR. The expression of GCS and mdr1 mRNA in clinic multidrug resistance samples exhibited significantly increased compared with clinic drug sensitive group ($P < 0.05$). There was the positive correlation both the expression of GCS and mdr1 genes in leukemia samples ($P < 0.01$, $\gamma = 0.7$). AlamarBlue Assay showed that the K562/A02 cell line was 115-fold more resistant to adriamycin and 36-fold more resistant to vincristine compared with drug-sensitive K562 cell line. There also was significant expression difference of GCS and mdr1 genes between K562 and K562/A02 cells. Bcl-2 gene exhibited higher expressions whatever in clinic drug-resistance samples or K562/A02 cells, whereas the expressions of Bax gene were higher in drug-sensitive samples and K562 cells. PPMP increased sensitivity to adriamycin toxicity by inhibiting GCS in K562/A02 cells. Therefore, it is suggested that a high level of GCS in leukemia is possible contributed to multidrug resistance of leukemia cells. Abnormally expressions of the genes in associated with cell apoptosis might be one of the main molecular pathology mechanisms of multidrug resistance caused by GCS gene.

Keywords: Glucosylceramide synthase; Leukemia; Drug resistance; Apoptosis; Bcl-2; Bax; PPMP

1. Introduction

Multidrug resistance (MDR) to chemotherapeutic agents is a major concern in patients with leukemia. In the classic model of multidrug resistance, a membrane-resident glycoprotein, termed P-glycoprotein that is phenotype of mdr1 gene, acts as a drug efflux pump, lowering intracellular drug levels to sublethal concentrations [1,2]. Other causes of multidrug resistance include overexpression of multidrug resistance-associated protein (a second drug efflux pump that is similar to P-glycoprotein) and modifications in glutathione *S*-transferase activity, etc., were also studied [3–5]. But, the clinical implications of a drug-efflux system are still controversial in patients with leukemia [6–8]. Robertson et al. [9], showed that some cells still survive even if anticancer drugs reach their sites of action. Studies [10–12] suggested that the dysfunctional metabolism of ceramide may contribute to multidrug resistance.

Ceramide is a basic unit of the lipid sphingomyelin. The production of ceramide is the result of diverse stimuli that include growth factor deprivation, cytokines, ionizing radiation, heat shock, chemotherapy and other cytotoxic agent, and various environmental factors. These stimuli ini-

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tiate ceramide-mediated signaling pathways [13]. Ceramide now has been recognized to contribute to cell cycle arrest, terminal cell differentiation, and apoptosis [14]. Loss of ceramide production is one cause of cellular resistance to apoptosis induced by either ionizing radiation or tumor necrosis factor-α and adriamycin [11]. Glucosylceramide synthase (GCS) is a glycosyltransferase in sphingolipid metabolism. This enzyme transfers a glucose residue from UDP-glucose to ceramide for synthesis of glucosylceramide (GC). Accumulation of GC is a characteristic of some MDR cancer cells and tumors derived from patients who are less responsive to chemotherapy [15]. The study of Liu et al. indicated that a decrease of the ceramide content was found in drug-resistant breast cancer MCF-7 cells because of enhancement of the activity of GCS, and overexpression of GCS by gene transfection conferred cellular resistance to chemotherapy [11,16]. Itoh et al. [17] recently also showed that overexpressing GCS significantly inhibited doxorubicin (adriamycin)-induced ceramide increase and apoptosis in HL-60/GCS cells. In our study, it was found that there was overexpression of GCS gene not only in multidrug-resistant K562/AO2 cells but also in the clinic chemoresistant cases with leukemia.

2. Materials and methods

2.1. Patient materials

Sixty-five of the patients with leukemia were admitted to No.1 hospital of Wuxi from 2002 to 2005. In a total of 65 cases, it was included 22 cases of AML, 18 cases of ALL, and 25 cases of CML. After two course of chemotherapy, based on the following criteria for CR: marrow with $\leq$5% leukemic blast cells concomitant with a neutrophil count of $\geq 1.5 \times 10^9\,L^{-1}$, 65 cases were subgrouped into drug-sensitive group in which the patients got into a CR state or drug-resistant group (non-CR). The peripheral blood cells were collected before chemotherapy. By density gradient centrifugation (Ficoll) mononuclear cells were collected and frozen at −80 °C until the following test was performed.

2.2. Cell cultures

The K562 and K562/A02 human leukemia cell lines were kindly provided by The Haematology Institute of Chinese Academy of Medical Sciences in Tianjin. The cells were maintained in RPMI 1640 medium (Sigma Co. Ltd., USA) containing 10% (v/v) FBS (Sigma Co. Ltd., USA), and 1 μg/ml adriamycin (Shanghai Hua Lian Co., China) was added in maintaining components for K562/AO2 cells. K562 and K562/AO2 cells were cultured in a humidified 5% CO_2 culture incubator at 37 °C.

2.3. Cytotoxicity assays

The cells were seeded in 96-well plates (1×10^4 cells/100 μl/well) and cultured in RPMI 1640 medium containing 10% (v/v) FBS at 37 °C for 6 h before addition of adriamycin or vincristine (Hua Lian Co., Shanghai, China). Drugs were added in FBS-free medium, and cells were cultured continually at 37 °C for 48 h. Drug cytotoxicity was tested by AlamarBlue Assay (Biosource Co., USA). The AlamarBlue Assay was performed as the protocol. Briefly, 25 μl AlamarBlue indicator was added to each well and cells were incubated for an additional 3 h. Then plates were read spectrophotometrically (absorbance at 570 and 600 nm). Cell viability (% of control) was calculated according to the formula:

$$\frac{117.216 \times A\lambda_1 - 80.586 \times A\lambda_2}{155.677 \times A'\lambda_2 - 14.652 \times A'\lambda_1} \times 100\%$$

A is the absorbance of test wells, A' is the absorbance of negative control wells, $\lambda_1 = 570$ nm, and $\lambda_2 = 600$ nm.

2.4. Chemical inhibition of glucosylceramide synthase

D,L-Threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), which is a chemical inhibitor for GCS, was from Sigma Co. (USA). To determine the relationship between GCS and drug-resistance of leukemia cells, K562/A02 cells were preincubated with PPMP (25 μM, 24 h), then adriamycin was added. After 48 h, drug cytotoxicity was tested as above.

2.5. RNA extraction and RT-PCR mRNA analysis

Total RNA of blood cells from clinic samples with leukemia, and RNA of K562 and K562/A02 cells were isolated using TRIzol reagent (Invitrogen GIBco Co.). Random primer (Shanghai Sangon Co., China) was used for reverse transcript reaction at 37 °C for 60 min. PCR primers sequences for GCS, mdr1, Bcl-2, Bax and β-actin as follows: GCS upstream 5′-CTGGAAACATTCTTTGAATTGGAT-3′ and downstream 5′-CTCATTAAACAAGACATTCCTGTC-3′; mdr1 upstream 5′-CTGGTGTTTGGAGAAATGACAG-3′ and downstream 5′-CCCAGTGAAAAATGTTGCCATTGAC-3′; Bcl-2 upstream 5′-GGATTGTGGCCTTCTTTGAG-3′ and downstream 5′-CCAAACTGAGCAGAGTCTTC-3′; Bax upstream 5′-TGCTTCAGGGTTTCATCCAGG-3′ and downstream 5′-TGGCAAAGTAGAAAAGGGCGA -3′; β-actin upstream 5′-GTGGGGCGCCCCAGGCACCA-3′ and downstream 5′-CTCCTTAATGTCACGCACGATTTC-3′. DNA was amplified in a 30-cycle PCR reaction, using the following conditions: denaturation at 94 °C for 60 s, annealing at 58 °C for 45 s (at 60 °C 45 s for Bcl-2), and elongation at 72 °C for 45 s. RT-PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. The fragment size of PCR products was 421 bp for GCS,