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ScienceDirect
Life Sciences 81 (2007) 1638–1644
Life Sciences
www.elsevier.com/locate/lifescie

Enhancing effects of ceramide derivatives on 1,25-dihydroxyvitamin D_3-induced differentiation of human HL-60 leukemia cells

Dong Soo Kim [a], Seung Hyun Kim [a], Ju Han Song [a], Young-Tae Chang [b], Seung Yong Hwang [c], Tae Sung Kim [a,*]

[a] School of Life Sciences and Biotechnology, Korea University, Anam-dong, Seoungbuk-Gu, Seoul 136-701, Republic of Korea
[b] Department of Chemistry, New York University, New York, NY10003, USA
[c] Division of Molecular and Life Sciences, Hanyang University, Ansan 426-791, Republic of Korea

Received 16 June 2007; accepted 13 September 2007

Abstract

Differentiation-inducing therapy by agents such as 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] represents a useful approach for the treatment for cancer, including acute myeloid leukemia (AML). Recent studies demonstrated that the combined administration of 1,25-$(OH)_2D_3$ and differentiation-enhancing agents could alleviate the side effects of 1,25-$(OH)_2D_3$ and improve the rate of long term survival. In this study, we determined the enhancing activities of ceramide derivatives on 1,25-$(OH)_2D_3$-induced differentiation of human myeloid leukemia HL-60 cells. Importantly, some of these derivatives—namely, A2, B3, and H9—enhanced the 1,25-$(OH)_2D_3$-induced differentiation of HL-60 cells in a concentration-dependent manner. In addition, the morphologic studies using Giemsa staining and flow cytometric analysis demonstrated that the combined treatment of 1,25-$(OH)_2D_3$ with one of the three analogues, A2, B3, and H9, directed the HL-60 cells into monocytic lineage, but not into granulocytic lineage. The inhibition studies demonstrated that A2, B3, and H9, enhanced 1,25-$(OH)_2D_3$-induced differentiation of HL-60 cells via the PI3-K/PKC/JNK/ERK pathways. The ability of ceramide derivatives to enhance the differentiation-inducing potential of 1,25-$(OH)_2D_3$ may contribute to an effective therapy for AML.
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Keywords: Ceramide library; Differentiation; Leukemia; 1,25-Dihydroxyvitamin D_3

Introduction

AMLs are malignancies that are raised by the clonal expansion of cells abnormally arrested at some maturation stages of hematopoietic precursors. Although differentiation-inducing therapy by several agents, such as all-*trans* retinoic acid (ATRA) and 1,25-$(OH)_2D_3$, induced favorable outcomes for patients with APL, the side effects, including recurrent disease and hypercalcemia, can be a major obstacle to disease-free survival. The HL-60 cell line originated from a patient with AML, and has been frequently employed as an excellent model line for the *in vitro* study of cellular differentiation because of its property that allows to differentiation into either monocytic or granulocytic lineages according to inducing stimuli (Collins, 1987).

The natural active form of vitamin D_3, 1,25-$(OH)_2D_3$, induces differentiation of normal and leukemic myeloid cells along the monocyte/macrophage lineage and inhibits proliferation of HL-60 cells in *in vitro* studies (Abe et al., 1981; McCarthy et al., 1983). *In vivo*, the survival of mice challenged with syngeneic leukemic cells was prolonged by treatment with 1,25-$(OH)_2D_3$. However, some of the patients treated with 1,25-$(OH)_2D_3$ underwent hypercalcemia at a serum level of 2×10^{-7} M, resulting in severe diseases such as a kidney failure and coma (Pakkala et al., 1995). This unexpected outcome points to additional trials, which can achieve both a greatest efficacy and a low side effect after treatment. Among challenges to these aims, combined treatments of natural products with 1,25-$(OH)_2D_3$ have been regarded as a useful therapeutic approach for achievement of a favorable outcome (Kim and Kim, 2002; Kim et al., 2002). Our group and other researchers have showed that some natural products and synthetic compounds can enhance the differentiation of HL-60 cells in combination

* Corresponding author. Tel.: +82 2 3290 3416; fax: +82 2 3290 3921.
E-mail address: tskim@korea.ac.kr (T.S. Kim).

0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.09.035

with low doses of ATRA or 1,25-$(OH)_2D_3$ (Beere and Hickman, 1993; Kang et al., 2001; Kim et al., 2003).

Ceramide is one of these natural products that show potential as an anticancer agent (Aouali et al., 2005). It represents the basic building block of sphingolipids which are abundant in membranes and are recognized as important lipid signaling mediators. Ceramide is synthesized by the *de novo* pathway and by the hydrolysis of plasma membrane sphingomyelin and more complex sphingolipids (Woodcock, 2006). Ceramide has a number of important physiologic functions which regulate cellular homeostasis, including regulation of the stress response, induction of cell differentiation, regulation of the cell cycle, and apoptotic cell death (Ogretmen and Hannun, 2001). However, its association with the differentiation of hematopoietic cells is not clear yet.

In this report, we assessed the enhancing effects of ceramide derivatives on the cellular differentiation of human myelocytic leukemia HL-60 cells, in combination with a low concentration of 1,25-$(OH)_2D_3$.

Materials and methods

Materials

The HL-60 cell line was initially obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). 1,25-$(OH)_2D_3$, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl) amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122), 2-[4-morpholinyl]-8-phenyl-1[4*H*]-benzopyran-4-one (LY 294002), anthrapyrazolone (SP 600125), Giemsa staining solution, methanol-free paraformaldehyde, and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Bisindolylmaleimide (GF 109203X), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) and 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) were purchased from Tocris Cookson Ltd (UK). A stock solution of 1 mM 1,25-$(OH)_2D_3$ was dissolved in absolute ethanol. The ceramide derivatives were dissolved in dimethyl sulfoxide to generate a 2 mM stock solution. The solutions were diluted at least 1000-fold in the growth medium, such that the final dimethyl sulfoxide concentration had no effect on differentiation and proliferation of the HL-60 cells. All manipulations were conducted under subdued light conditions.

Synthesis of ceramide derivatives

The ceramide derivatives were synthesized as previously described (Park et al., 2005). In brief, an activated ester resin (50 mg, 75 μmol) was added to a core amine (3 μmol) in THF (1 mL) and agitated on a shaker overnight at room temperature. The reaction mixture was filtered and washed with a portion of THF (1 mL), and the filtrate was combined and dried. Completion of the reaction was confirmed by TLC and negative ninhydrin staining. The purity of each compound was checked by TLC and phosphomolybdic acid staining; each gave a single spot.

Determination of HL-60 cell differentiation

HL-60 cell differentiation was assessed via a nitroblue tetrazolium (NBT) reduction assay, as described previously (Kim et al., 2001). This assay is based on the ability of phagocytic cells to generate superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested via centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS, containing 200 ng/mL of freshly diluted PMA at 37 °C for 30 min in darkness. Cytospin slides were prepared and examined for blue–black nitroblue diformazan deposits, which are an indicative of a PMA stimulated respiratory burst. At least 200 cells were assessed in each experiment.

Morphologic studies

Single-cell suspensions were prepared and 2×10^5 cells were loaded into a cytofunnel and spun at 500 rpm in a cytospin centrifuge. The slides were fixed with methanol and dried. The slides were then stained for 20 min with Giemsa staining solution, rinsed in deionized water, air-dried, and observed under a microscope equipped with a camera. The stained cells were assessed with regard to size, regularity of the cell margin, and morphological characteristics of the nuclei.

Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were conducted using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and analyzed with CELLQuest™ software. In brief, single-cell suspensions were collected from the various cultures and washed twice in ice-cold phosphate-buffered saline (PBS, pH 7.4). Afterwards, phycoerythrin (PE)-conjugated anti-human CD11b or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA) were added, followed by 1 h of incubation at 4 °C. After incubation, the cells were washed with PBS and fixed in PBS containing 1% paraformaldehyde, and cytofluorometric analysis was conducted. Background staining was performed via the staining of the cells with PE- or FITC-conjugated isotype control monoclonal antibodies. One-parameter fluorescence histograms were generated via the analysis of at least 1×10^4 cells.

Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA), followed by the Bonferroni method were used to determine the statistical significance of differences between the values of various experimental and control groups. A *P*-value of <0.05 was considered to be significant.

Results

Synthesis of ceramides

Ceramide derivatives were synthesized by solid phase combinatorial chemistry as previously described (Chang et al.,

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