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Parthenolide Induces Apoptosis in Committed Progenitor AML Cell line U937 via Reduction in Osteopontin

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Abstract

Background: Interfering with cell proliferation and survival is a critical role for antineoplastic drugs leading to cell death through induction of apoptosis. Alternative treatments with herbal extracts offer insights into acute myeloid leukemia (AML) therapy. Parthenolide (PTL), an extract from feverfew, induces apoptosis in primary human leukemia stem cells (LSCs) and bulk leukemic cell populations. Osteopontin (OPN) preserves cell viability in response to anticancer agents and its receptors could be utilized for therapeutic targeting of cancer cells.

Methods: U937 cells were cultured in RPMI 1640 with concentrations of 2, 4, 6, 8, and 10 µM PTL for 20-24 hours for MTT assays. Apoptosis assays were performed with Annexin V-Alexa Fluor-488/PI as Annexin V+/PI- and Annexin V+/PI+ to measure early and late apoptosis, respectively. Quantitative real-time PCR was used to measure OPN gene expression using the $2^{-\Delta\Delta Ct}$ method. The PTL–treated cells were stained with FITC-CD38 antibody for flow cytometry analyses. Data were compared using one-way analysis of variance (ANOVA) by SPSS 19.

Results: Parthenolide inhibited growth of U937 cells with IC25 and IC50 values of 4 and 5.8 µM, respectively. Death induction with PTL was apoptotic. Flow cytometry showed a significant decrease in the percentage of CD38+ U937 cells in response to PTL. Osteopontin gene expression decreased in response to PTL.

Conclusions: PTL induced apoptosis and reduced OPN gene expression in U937 cells.

Keywords: AML cell line U937, Osteopontin, Parthenolide

Introduction

Acute myeloid leukemia (AML) is a clonal disorder through transformation and uncontrolled proliferation of myeloid progenitor cells with arrested differentiation (1). Leukemic stem cells (LSCs) are AML-initiating cells of various populations with different features (2, 3). Acute myeloid leukemia-initiating cells are identified immunophenotypically as CD34+ and CD38- or CD34+ and CD38+ (4, 5). As AML cells mature, CD34 expression decreases gradually while CD38+ increases (6, 7). Current AML treatment utilizes chemotherapy with cytarabine and an anthracycline to achieve complete remission (CR) (8). Most therapies target molecules involved in

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the cell cycle; therefore, proliferation of AML cells can be affected (9). Interfering with cell proliferation and survival through induction of apoptosis is a critical. One problem with conventional AML therapies is unwanted side effects. Alternative treatments with herbal extracts could both increase efficiency in molecular targeting and have fewer unwanted side effects.

Parthenolide (PTL), an extract from feverfew, induces apoptosis in primary human LSCs and bulk leukemic cell populations (10-12). NF-kB inhibition is the best-known mechanism of PTL-induced apoptosis (13).

Osteopontin (OPN) is a glycoprotein expressed by cells in many tissues. Osteopontin preserves cell viability in response to anticancer agents and its receptors could be utilized for therapeutic targeting of cancer cells (14, 15). Osteopontin exists as both a secreted (sOPN) and intracellular (iOPN) protein. Osteopontin is recognized by a variety of integrins including αvβ3 and CD44 (16). In the present study, we evaluated the role of OPN in U937 cell viability after PTL treatment.

Materials and Methods

Materials

Parthenolide was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) as a 50mM stock solution, stored at -20 °C, and diluted in DMSO before use. The Annexin V-Alexa Fluor-488/PI kit and the human monoclonal antibody FITC anti-CD38 were purchased from BD Biosciences (San Jose, CA, USA). TriPure Isolation Reagent was purchased from Roche Applied Science (Germany). The cDNA synthesis kit and SYBR® Premix Ex Taq™ were purchased from Takara Biotechnology Co. (Otsu, Japan).

Cell culture

The human leukemic monoblast U937 cell line was obtained from the Pasteur Institute of Iran. RPMI 1640-10% fetal bovine serum (FBS) medium (Gibco; Invitrogen, USA) was used for culturing. The medium was supplemented with 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT assay

Cells were cultured in triplicate at 5×10^3/100µl in 96-well culture plates (SPL Life sciences, Pocheon, Korea) with 2, 4, 6, 8, or 10 µM PTL for 20 to 24 hours.

After incubation at 37 °C in 5% CO_2, the cells were incubated for 4 h with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, 5mg dissolved in 1 ml of PBS, Sigma, St. Louis, MO, USA). The plates were centrifuged (10 min at 350×g), and the purple formazan crystals of metabolized yellow tetrazolium salt by viable cells were dissolved in DMSO. Absorbance was quantified at 570 nm using the ELISA plate reader (Microplate Reader; Bio-Rad). Results were expressed as a percentage of viability, with 100% representing control cells treated with 0.1% DMSO alone.

Evaluation of apoptosis by annexin V/Propidium iodide (PI) assay

Cells were cultured at 1×10^6/ml per well in 6-well plates treated with PTL as above. After 24 hours cells were harvested and mixed with Annexin V-Alexa Fluor-488/PI according to the manufacturer's instructions. The stained cells were examined by flow cytometry (Partec, Munich, Germany). Discrimination of cells was performed as apoptosis (Annexin V^+/PI^- [early apoptosis] and Annexin V^+/PI^+ [late apoptosis]).

Flow cytometry

Cells were treated with PTL for 24h. Before labeling, the cells were centrifuged (10min at 200×g) to remove the debris and resuspended in PBS. Then, the cells were stained with the human monoclonal antibody FITC anti-CD38. An appropriate isotype control IgG1 was used. The cells were analyzed on a Partec PAS III flow cytometer (Partec, Munich, Germany), and data were interpreted using FloMax software.

Quantitative Real-time PCR (qRT-PCR)

Total RNA of the PTL-treated and untreated cells was extracted with TriPure Isolation Reagent

according to the manufacturer's instructions, quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and stored at −80 °C. The cDNA synthesis kit was used to synthesize complementary DNA (cDNA). A light cycler instrument (Roche Diagnostic, Manheim, Germany) and SYBR Premix Ex Taq were used for quantitative real-time RT-PCR analysis. A final volume of 20 µl containing 2 µl of a 2-fold diluted cDNA, 1 µl of 10 pmol primers (0.5 µl each forward and reverse primers), 10 µl of SYBER, and 7 µl of distilled water were used. Data were normalized to HPRT expression in each sample. Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Table 1 shows the primer sequences for genes used.

Statistical analysis

Using IBM SPSS Statistics 19 software the groups of data was presented as means ±SDs and compared by one-way analysis variance (ANOVA) or t-test.

Table 1. Primers used for qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)
HPRT	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
OPN	ACCCTTCCAAGTAAGTCCAACG	GGTGAGAATCATCAGTGTCATCTAC	139

Results

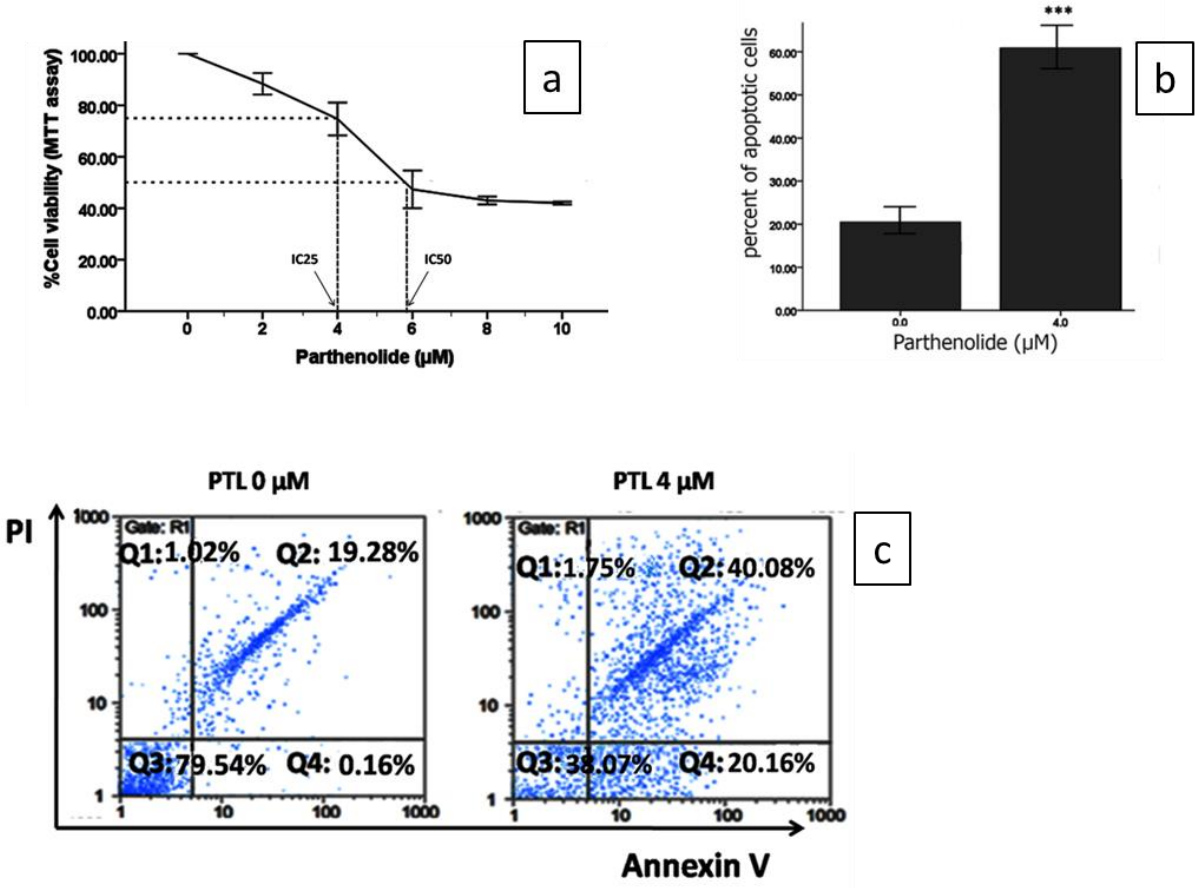

Fig. 1. Parthenolide induced apoptosis in U937 cells (a) Dose-response curves with different concentrations of PTL using MTT assay/24 h were generated. (b) The performance of the Annexin-V/PI staining on treated cells with PTL. (c) Representative dot-plot diagrams of AV/PI flow cytometry. The graphs represent three independent experiments (mean±SD). *P<0.05, **p<0.01, ***p<0.001 (compared with control or comparisons depicted).

Parthenolide inhibited cell growth of U937 cells

Dose-response curves with increasing concentrations of PTL using the MTT assay at 24 h showed that 6 µM PTL decreased U937 cells viability to 47%, while other concentrations of PTL did not significantly affect cell viability (Fig. 1a). The IC25 and IC50 values at 24 h were 4 and 5.8 µM respectively. The 4 µM concentration was chosen for the other assays.

Parthenolide induced apoptosis in U937 cells

Annexin-V/PI staining indicated that PTL induced apoptosis. The average percentage of apoptosis was 58.51 ±2.1 within 24 h (Fig. 1b).

Parthenolide reduced the CD38+ population of U937 cells

Flow cytometry analysis (Fig. 2) showed a significant decrease in the percentage of the CD38+ population from 43.4 ±2.3% in untreated cells to 3.5±1.5% in response to PTL ($p < 0.001$).

Parthenolide decreased OPN expression in U937 cells

We investigated OPN mRNA levels by quantitative RT-PCR to determine the effects of PTL on this reported chemotherapy resistance factor. Osteopontin gene expression was significantly less in the PTL-treated than in the untreated cells ($p<0.001$, Fig. 3).

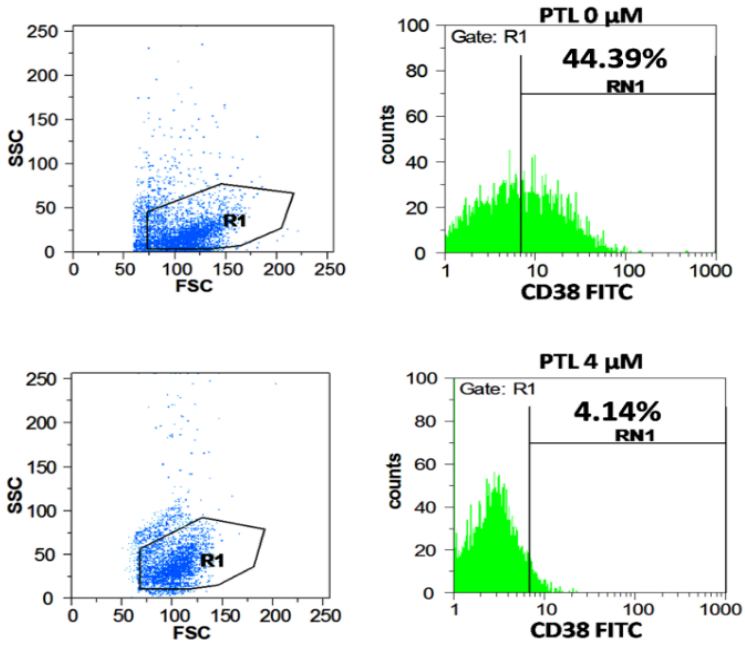

Fig. 2. Parthenolide reduced the CD38+ population of U937 cells. Flow cytometric analysis of PTL-treated/24 h U937 cells stained with monoclonal antibody FITC-CD38.

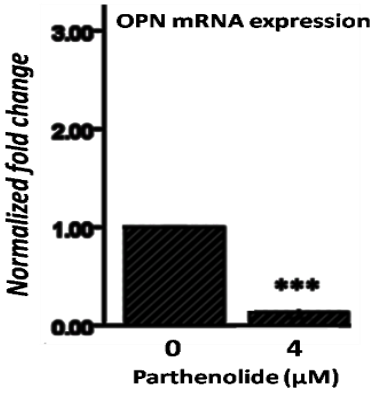

Fig. 3. Parthenolide decreased OPN expression in U937 cells. Evaluation of mRNA expression of OPN relative to HPRT, using real time RT-PCR after treatment of U937 cells with PTL for 24h. Three independent experiments were performed (mean ± SD). *P < 0.05, **p < 0.01, ***p < 0.001 (compared with control).

Discussion

Our data indicated that U937 AML cells are sensitive to PTL. The observed decrease in OPN expression could play a role in this sensitivity. Parthenolide has been used for centuries against fever and inflammation. In addition, PTL effects on cancers have been studied. Osteopontin has been shown to mediate anti-apoptotic effects on cancer cells in response to cytotoxic agents (16).

In recent years, PTL has been shown to eradicate LSCs (11, 12). Given that AML consists of stem and bulk AML cells, therapies targeting both populations would be ideal. However, due to differences between the two populations, most therapies can only be directed at one of them (9). Leukemic stem cells are immunophenotypically $CD34^+$ and $CD38^-$ while more mature cells are $CD34^+$ and $CD38^+$ (2). When CD34 gradually decreases, CD38 increases on committed progenitors (6, 7). Whereas U937 cells express committed progenitor surface markers (17), Parthenolide could affect AML bulk cells. PTL treatment of bone marrow cells in children with acute lymphocytic leukemia has shown that the CD34+ subpopulations are more resistant to PTL than the CD34- subpopulations (18). In clinical settings, a PTL water-soluble analog called dimethylamino-parthenolide (DMAPTL) has been utilized that kills both LSCs and bulk leukemic cell populations (19).

Parthenolide induces apoptosis in cancer cells by interfering with the function of vital proteins in gene transcriptional regulatory units, especially with NF-kB, as well as via other mechanisms such as PI3K pathway suppression, reactive oxygen species induction, and stress response proteins activation (20-26). In our work PTL decreased OPN transcription. A NF-kB binding site on the OPN promoter has been identified that regulates OPN expression (27, 28). A blocking role of PTL in U937 cells on NF-kB, which involves IL-6 gene expression, as an apoptosis inhibitory stimulus in some hematologic malignancies, has been reported (29-31). AP-1, another OPN transcriptional activator, is also inhibited by PTL (32, 33). In conclusion, PTL inhibits OPN expression and induces apoptosis in U937 cells. Thus, PTL could be considered as a therapeutic tool and OPN as a target molecule to eradicate bulk AML cells.

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