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Enhancement of caffeic acid phenethyl ester on all-trans retinoic acid-induced differentiation in human leukemia HL-60 cells

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Abstract

All-trans retinoic acid (ATRA) induces complete remission in a high proportion of patients with acute promyelocytic leukemia (APL); however, the response is sometimes very slow. Furthermore, relapse and resistance to treatment often occur despite continued treatment with ATRA. Thereafter, combination treatment strategies have been suggested to circumvent these problems. The present study demonstrates that caffeic acid phenethyl ester (CAPE), a major component of honeybee propolis, enhanced ATRA-induced granulocytic differentiation in HL-60, a human promyelocytic cell line. The differentiation was assessed by Wright–Giemsa stain, nitroblue tetrazolium reduction, and membrane differentiation marker CD11b. In addition, CAPE enhanced ATRA-induced cell cycle arrest at the G1 phase by decreasing the association of cdk2-cyclin E complex. Finally, it was demonstrated that CAPE promoted the ATRA-mediated nuclear transcription activation of RARα assessed by EMSA assay and enhanced the expression of target genes including RARα, C/EBPε, and p21 protein resulting in the differentiation development of leukemia. It is suggested that CAPE possesses the potential to enhance the efficiency of ATRA in the differentiation therapy of APL.

Keywords: Caffeic acid phenethyl ester; All-trans retinoic acid; Differentiation human leukemia HL-60 cells

Introduction

Caffeic acid phenethyl ester (CAPE), one of the significant components of honeybee propolis, has been identified to possess varied biochemical responses including anti-oxidant, anti-viral, anti-inflammatory properties, as well as selectively repressing the proliferation of several carcinoma cells but not untransformed cells (Fesen et al., 1994; Natarajan et al., 1996; Michaluart et al., 1999; Frenkel et al., 1993). Our previous studies showed that CAPE exhibited significant cytotoxicity in oral cancer cells (Lee et al., 2000). In addition, CAPE induced apoptosis of C6 glioma cells in concentrations above 50 μM and was mediated via signaling pathway of p38 mitogen-activated protein kinase (p38 MAPK) (Lee et al., 2003). From in vitro and in vivo studies, we found that CAPE demonstrated a growth inhibitory effect on C6 glioma cells (Kuo et al., 2006). Recently, CAPE has been shown to exert anti-leukemia properties (Chen et al., 1996) but the effects on the induction of differentiation are still unknown.

All-trans retinoic acid (ATRA) belongs to a class of chemical compounds structurally related to vitamin A and is one of the classes of compounds known as retinoids. Some of these compounds, including vitamin A, have shown limited success in the prevention of cancer and anti-cancer therapy. The mechanism of action of retinoids in chemoprevention and therapy of cancers involves modulation of cell proliferation and differentiation (Freemantle et al., 2003; Altucci and Gronemeyer, 2001; Ross et al., 2000). It was generally accepted that one or more of the

Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; CAPE, caffeic acid phenethyl ester; EMSA, electrophoretic mobility shift assay; NBT, nitroblue tetrazolium; RAR, retinoic acid receptor; RARE, retinoic acid response elements.

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nuclear receptors probably mediates the actions of RA in cell differentiation (Linney, 1992). Activation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are the members of the steroid/thyroid hormone receptor superfamily of nuclear transcription factors, is associated with the differentiation transcription of a variety of genes. ATRA and 9-cis RA are considered as the most potent modulators of hematopoiesis. Whereas RXRs binds the 9-cis RA with a high affinity, RARs bind both ATRA and 9-cis RA. The three types of RARs, α, β, and γ, and the three types of RXRs, α, β, and γ, are ligand-inducible transregulators that modulate the transcription of target genes by forming dimmers and interacting with *cis*-acting DNA retinoic acid response elements (RAREs) (Pitha-Rowe et al., 2003). It is well known that ATRA induces differentiation of HL-60 cells to granulocyte mediated by RAR (Robertson et al., 1992). On the other hand, the role of RXR ligand during HL-60 differentiation was thought to be a synergistic enhancement of RAR activity (Apfel et al., 1995). However, the existence of a RAR-independent RXR pathway was reported (Ueno et al., 1998). In addition, it has been reported that RARα is directly involved in differentiation of HL-60 leukemia cells and that the dominant inhibitory form of RARα can prevent this differentiation process (Collins et al., 1990; Tsai et al., 1992).

Differentiation therapies in oncology are broadly defined as those that induced malignant reversion. Clinically, these therapies have been most successful in acute promyelocytic leukemia (APL), with the use of ATRA. Although the success obtained with APL patients has raised enthusiasm for the clinical use of ATRA in the treatment of leukemia and other neoplastic diseases, the therapeutic efficacy of this compound is still burdened by problems such as resistance and toxicity (Cornic and Chomienne, 1995; Delva et al., 1993; Muindi et al., 1992). One possible strategy to increase the therapeutic index of ATRA is the development of ATRA-based pharmacological combinations that are more powerful and easily tolerated than the individual component (Bruserud and Gjertsen, 2000). Because HL-60 cells provide a useful system for studying cellular and molecular events involved in differentiation by chemical agents, in the present study, we used the HL-60 cells to investigate the combination effect of CAPE and ATRA on differentiation. We found that CAPE enhances the ATRA-induced granulocyte differentiation and cell cycle arrest, which involves transcription activation of nuclear transcription factors such as RARα.

Materials and methods

Chemicals and antibodies. Caffeic acid phenethyl ester (CAPE) was supported by Dr. Lee YJ (Department of Chemistry, National Changhua University of Education, Changhua, Taiwan). ATRA, 1,25-dihydroxyvitamin D3 (1,25D_3), and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies against retinoid acid receptor (RARα and RARβ), retinoid X receptor (RXRα), C/EBPε, p21, CDK2, cyclin E, β-actin, and anti-rabbit IgG-FITC, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phycoerythrin-conjugated anti-CD14 and fluorescein isothiocyanate-conjugated anti-CD11b antibodies were obtained from BD Biosciences-PharMingen (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were obtained from Santa Cruz Biotechnology Inc.

Cell culture and proliferation assay. The human HL-60 cell line was obtained from American Type Culture Collection. These cells were cultured routinely with RPMI-1640 medium (Gibco) supplemented with 1% glutamine and 10% heat-inactivated fetal calf serum (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were seeded at 4.0×10^5 cells/ml and incubated for 96 h with CAPE, ATRA, or combinations. Cell growth was estimated by trypan blue dye (0.25%) exclusion assay.

Staining and nitroblue tetrazolium (NBT) reduction assay. Prior to induction of differentiation by agent, HL-60 cells were maintained at a logarithmic growth rate and seeded at a density of 2×10^5 cells/ml. Following exposure to the indicated agent or combinations for 4 days, cells were collected by cytospin centrifugation and stained with Wright–Giemsa stain and observed by microscopy. In addition, the treated cells were pelleted by centrifugation at 300×*g* for 5 min. Differentiation of HL-60 cells was measured by adding 1 ml of cell suspension (1×10^5 cells) to a solution containing 2 mg/ml of NBT and 20 ng/ml of phorbol myristate acetate in phosphate-buffered saline. The incubation was allowed to proceed for 1 h at 37 °C and was stopped by the addition of 0.4 ml of cold 2 M HCl. The formazan product was obtained by centrifugation of the sample at 700 × *g* for 10 min. The supernatant was discarded, and the formazan was dissolved in 1 ml of DMSO. The absorbance of the solution was measured at 590 nm. The data are expressed as percentage of control absorbance units.

Determination of differentiation markers. HL-60 cells were plated at an initial density of 4.0×10^5 cells/ml in the presence of the indicated agents or combinations. Following 4 days of culture, granulocytic/monocytic marker CD11b/CD14 were measured following staining with fluorescein isothiocyanate-conjugated anti-CD11b antibody or R-phycoerythrin-conjugated anti-CD14 antibody for 25 min at room temperature. The stained cells were washed twice with phosphate-buffered saline (PBS) containing 2% bovine serum albumin. The percentage of cells with fluorescence intensity was measured using a FACS Calibur flow cytometer with CELL Quest software (Becton Dickinson).

Cell cycle distribution analysis. Flow cytometric analysis of the HL-60 cells was performed by a FACScan (Becton Dickinson Immunocytometry Systems, UK). Following exposure to indicated agents or combination for 4 days, cells (1.0×10^6) were collected in 0.2 ml of 1% FBS in PBS and fixed by adding 0.8 ml of ethanol (−20 °C; 100%) dropwise with continuous vortexing. After overnight incubation at −20 °C, cells were pelleted and resuspended in trypsin inhibitor, and RNase buffer was added at the room temperature. Prior to the analysis of flow cytometry, cold propidium iodide (PI) stain solution (1 ml) was added to the mixture, and it was incubated at room temperature for 10 min. The DNA content of the stained nuclei was analyzed by flow cytometry. The distribution of DNA content was expressed as G1, S, and G2/M phases.

Preparation of total cell extracts and immunoblots analysis. Cells were plated onto 10 cm^2 dishes at a density of 4×10^5 cells/ml with or without CAPE and harvested. To prepare the whole-cell extract, we washed cells with PBS plus zinc ion (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinine, 170 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.5). After being mixed for 30 min at 4 °C, the mixtures were centrifuged (10,000 × *g*) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as the standard. The ECL western blotting was performed as follows. An equal protein content of total cell lysates from the control and treated samples was resolved on 10–12% SDS–PAGE gels along with prestained protein molecular weight standards (Bio-Rad). Proteins were then blotted onto NC membrane (Millipore) and reacted with the primary antibodies. The secondary antibody was a peroxidase-conjugated goat anti-mouse antibody. After binding, the bands were revealed by enhanced chemiluminescence with the ECL commercial kit quantitative assay (Fujifilm Image Gauge program).

Immunoprecipitation assay. To detect association of cdk2/cyclin E, we washed cells twice with ice-cold PBS and lysed in lysis buffer. The lysates were vortexed and cleared by centrifugation at 10,000 × *g* for 10 min. The lysates were immunoprecipitated using monoclonal anti-cdk2 (1 μg) and 15 μl of protein A/G-agarose (Santa Cruz) at 4 °C for 4 h. The eluates from the beads