Journal of Life Science 2007 Vol. 17. No. 9. 1191~1196 ©JLS / ISSN 1225-9918

Cooperative Induction of HL-60 Cell Differentiation by Combined Treatment with Eugenol and 1α,25-Dihydroxyvitamin D_3

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Received July 25, 2007 / Accepted September 10, 2007

Eugenol (4-allyl-2-methoxyphenol) is a main component of essential oils obtained from various spices. Recent reports have shown that eugenol induces growth inhibition and apoptosis of malignant tumor cells. In this study, the stimulatory effect of eugenol on cell differentiation was investigated in HL-60 promyelocytic leukemia cells. When HL-60 cells were treated in combination with 150 μM of eugenol and 3 nM of 1α,25-dihydroxyvitamin D_3, cell growth was slower than that of cells treated with eugenol or 1α,25-dihydroxyvitamin D_3 alone. Eugenol enhanced low dose of 1α,25-dihydroxyvitamin D_3-induced a G_0/G_1 phase arrest in cell cycle. Consistent with this, combined treatment of eugenol and 1α,25-dihydroxyvitamin D_3 cooperatively increased p27 level and decreased cyclin A, cdk 2 and cdk 4 levels, which are cell cycle regulators related to G_0/G_1 arrest. According to flow cytometric analysis, the expression of CD14 (monocytic differentiation marker) was more increased in the cells co-treated with eugenol and 1α,25-dihydroxyvitamin D_3. These results indicate that eugenol potentiates cell differentiation mediated by 1α,25-dihydroxyvitamin D_3 of suboptimal concentration. The differentiation-inducing property of eugenol maybe contributes to chemopreventive activity of cancer.

Key words – Eugenol, 1α,25-dihydroxyvitamin D3, cell differentiation, cell cycle, HL-60 cells

Introduction

Conventional chemotherapy has a cytotoxicity for normal cells as well as cancer cells, and appears harmful side effects. One of the useful alternative approaches to cancer therapy is a differentiation therapy. In the differentiation therapy, unlike cytotoxic chemotherapy, gene transcriptions required for cell differentiation are regulated and immature cancerous cells are reprogrammed to undergo terminal differentiation without damages to the normal cells. Treatment for acute promyelocytic leukemia (APL) is a practical model of the differentiation therapy [9,18].

APL presents as an abnormal accumulation of promyelocytic cells caused by a failure in differentiation of immature cells towards mature cells [11]. As pharmacologically high doses of all-*trans* retinoic acid (ATRA) can overcome this failure and induce neutrophilic differentiation of the APL cells both *in vitro* and *in vivo*, differentiation-inducing therapy with ATRA has been used in APL patients [10,12]. Although ATRA therapy is effective in inducing remission, it has some problems that a few of patients relapse within 4-5 years and most of the relapsed APL patients are resistant to further treatment with ATRA [2,5].

Other differentiation-inducing agents, especially vitamin D_3 (vit D_3) derivatives, have been actively investigated. The major function of vit D_3 involves calcium homeostasis in the body. The active metabolite resulted from sequential hydroxylations at the 1α and 25 position, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2$ vit D_3) has been known as an inhibitor of cell proliferation and a stimulator of cell differentiation in a variety of malignant cells [1,8]. This biological response is mediated by binding to the nuclear receptor for $1,25(OH)_2$ vit D_3, which is a member of a superfamily of steroid hormone receptors and regulates the gene transcription by interacting with specific response elements in the promoter region of target genes [3,6,13]. However, the clinical trials of $1,25(OH)_2$ vit D_3 for leukemia have been limited because of its hypercalcemic side effect and incomplete differentiation [7]. Achieving terminal and irreversible differentiation and solving hypercalcemic problem are a major goal in therapeutic strategies using $1,25(OH)_2$ vit D_3. One of the possible strategies is to combine a low dose of $1,25(OH)_2$ vit D_3 with another drugs which potentiate the differentiation activity of $1,25(OH)_2$ vit D_3 [14-16].

Eugenol (Fig. 1) is found widely as a component of essential oils and is a major constituent of various spices such as clove oil, bay leaves and cinnamon leaf. The compound

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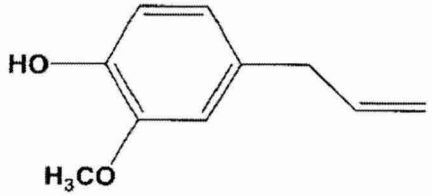


Fig. 1. Chemical structure of eugenol.

has been primarily used as a flavoring agent in a variety of foods and widely used as an antibacterial and analgesic agent in Asian traditional medicine. Recent reports demonstrated that the eugenol induced apoptosis of leukemia cells through a generation of reactive oxygen species [17], and suppressed growth of melanoma cells through an inhibition of E2F1 transcriptional activity that plays an important role in regulating cell cycle progression [4]. Because several cell cycle-regulating agents participate in the induction of cell differentiation as well as apoptosis, we wondered if the eugenol also possesses a differentiation-inducing activity on malignant cells. To investigate a cell-differentiating activity of eugenol, in this paper, we studied the stimulating effect of eugenol on HL-60 cell differentiation triggered by subeffective dose of $1,25(OH)_2$ vit D_3 and analyzed the regulation of cell cycle-related proteins in the cells treated with combination of eugenol and $1,25(OH)_2$ vit D_3.

Materials and Methods

Cell treatment

HL-60 cell line (human promyelocytic leukemia cell) was purchased from American Type Culture Collection (Rockvill, MD) and maintained in RPMI 1640 medium (Sigma; St. Louis, MO) supplemented with 10% fetal calf serum (HyClone; Logan, UT) at 37℃ in a humidified atmosphere of 5% CO_2. To induce differentiation, the cells were incubated with 150 μM eugenol (Tokyo Kasei Kogyo; Tokyo, Japan), 3 nM $1,25(OH)_2$ vit D_3 (Sigma) or a combination of eugenol and $1,25(OH)_2$ vit D_3 for 3 days.

Cell cycle analysis

The HL-60 cells were treated with 150 μM eugenol or 3 nM $1,25(OH)_2$ vit D_3 or with a combination of two drugs. After incubation for 3 days, the cells were washed with PBS twice, fixed in cold 70% ethanol and stood at 4℃ overnight. The fixed cells were resuspended in 1 ml of PBS containing 50 μg/ml propidium iodide (Sigma) and 0.1 mg/ml DNase-free RNase A (Boehringer Mannheim; Mannheim, Germany), and then incubated for 30 min at 37°C. After staining, cell cycle analysis was performed with a FACScan flow cytometer (BD Biosciences, San Diego, CA).

Flow cytometric analysis of CD 14 expression

A direct immunofluorescence staining technique was used to detect CD14 cell surface antigen. The cells, treated with eugenol or/and $1,25(OH)_2$ vit D_3 for 3 days, were washed twice with buffer A (PBS, 0.1% sodium azide, 1% heat-inactivated fetal bovine serum). After being resuspended in buffer A, an aliquot of cells (1×10^6 cells) was incubated with FITC-conjugated monoclonal mouse anti-human CD14 antibody (DAKO; Denmark) on ice for 30 min, and then analysed by a flow cytometer. For isotype control staining, an aliquot of cells was incubated with FITC-conjugated mouse IgG1 (DAKO). The incubated cells were washed again with buffer A, resuspended in 500 μl of buffer A containing propidium iodine, and then analyzed on a FACScan flow cytometer.

Western blot analysis

The drug-treated cells were washed twice with PBS, and then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml, pH 8.0). After determining the protein concentration of each lysate, equal amounts of the samples were loaded on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and detected using polyclonal antibodies against human cdk2, cdk4, cyclin A (Santa Cruz Biotechnology; Santa Cruz, CA), and p27 (BD Biosciences; Palo Alto, CA) and an enhanced chemiluminescence detection kit (Amersham; Buckinghamshire, U.K.)

Results and Discussion

Cooperative inhibition of the growth of HL-60 cells by eugenol and $1,25(OH)_2$ vit D_3

To determine the concentrations of eugenol without cytotoxicity in HL-60 cell culture, the cells were treated with 150 μM - 1200 μM of eugenol and cultured for 4 days. The cell growth was suppressed by eugenol in concentration range of 150 μM - 300 μM, while concentrations greater than 600 μM were cytotoxic (Fig. 2). Therefore, 150 μM of

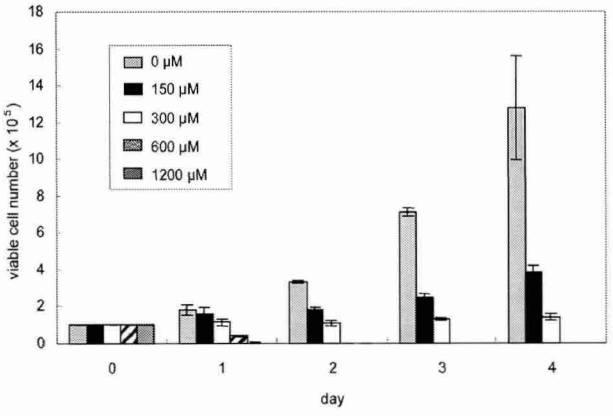


Fig. 2. The suppression of cell growth by eugenol. HL-60 cells were seeded in 6-well plate at a density of 1 x 10^5/ml. The cells were treated with various concentrations of eugenol (150 μM - 1200 μM) for 1-4 day. At the indicated timepoints, the number of viable cells was determined by use of a haemocytometer. Results were presented means ± S.D. of triplicate experiments.

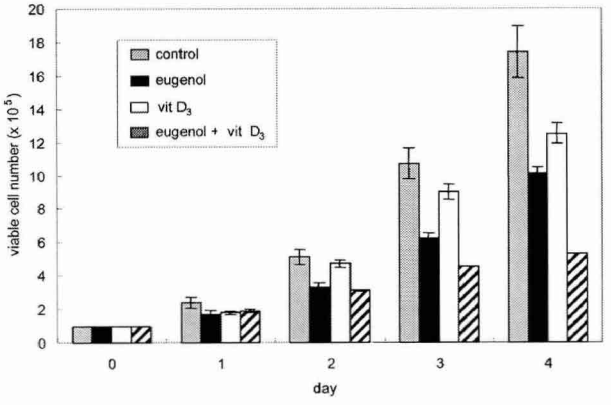
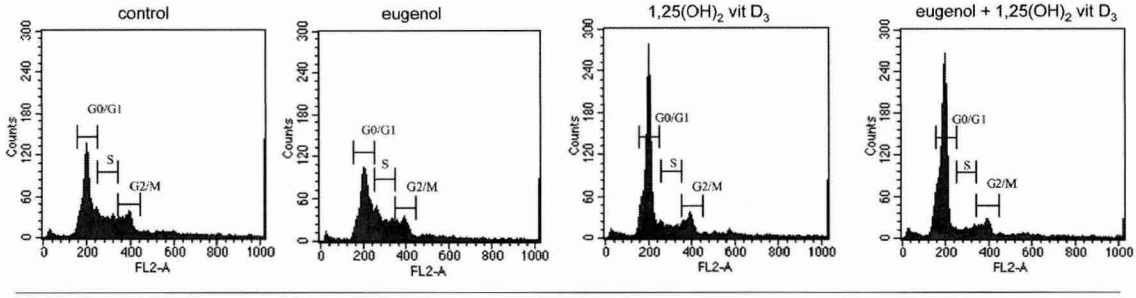


Fig. 3. The inhibitory effects of eugenol and 1,25(OH)$_2$ Vit D_3 on growth of HL-60 cells. HL-60 cells were seeded in 6-well plate at a density of 1 x 10^5/ml. The cells were treated with eugenol (150 μM) or 1,25(OH)$_2$ vit D_3 (3 nM) alone or in combination with both drugs for 1 - 4 day. At the indicated timepoints, the number of viable cells was determined by use of a haemocytometer. Results were presented means ± S.D. of triplicate experiments.

eugenol was used in this study. To investigate whether the eugenol cooperates with 1,25(OH)$_2$ vit D_3 in inhibiting cell growth, HL-60 cells were treated with the eugenol in the absence or presence of subeffective concentration (3 nM) of 1,25(OH)$_2$ vit D_3. After incubation for 1-4 day, the cell growth was examined. The growth of cells treated with eugenol and low dose of 1,25(OH)$_2$ vit D_3 in combination was slower than that of cells treated with eugenol or 1,25(OH)$_2$ vit D_3 alone (Fig. 3).

Regulation of cell cycle by cotreatment of eugenol and 1,25(OH)$_2$ vit D_3

To identify whether the retardation of cell growth is related to G_0/G_1 arrest in cell cycle, we investigated the effect of eugenol on cell cycle progression and the expressions of cell cycle regulators. Flow cytometric analysis of propidium iodide-stained nuclei showed that the eugenol significantly potentiated 1,25(OH)$_2$ vit D_3-mediated cell



Treatment	% Fluorescence at cell cycle Phase		
	G0/G1	S	G2/M
Control	50.77±4.05	22.33±2.51	16.14±0.86
Eugenol	49.55±4.61	26.91±3.04	14.59±2.41
1,25(OH)$_2$ vit D_3	66.73±6.62	10.89±0.63	11.5±1.29
Eugenol+1,25(OH)$_2$ vit D_3	76±7	8±0.26	9.56±0.45

Fig. 4. Cooperative induction of G_0/G_1 arrest by eugenol and 1,25(OH)$_2$ vit D_3. The cells were treated with 150 μM eugenol or 3 nM 1,25(OH)$_2$ vit D_3 alone or in combination with the two compounds. After incubation for 3 days, the cells were fixed in cold ethanol and incubated for 30 min at 4°C in a solution of 50 mg/ml propidium iodide, 1 mg/ml RNase and 0.1% NP-40. Cell cycle analysis was performed immediately after staining with a FACScan flow cytometer. Experiments were carried out in triplicate and the results represent means ± S.D. of data from three experiments.

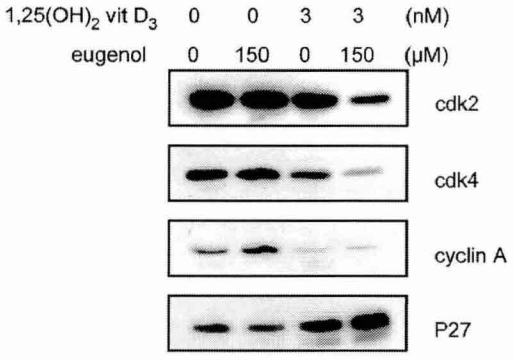


Fig. 5. Regulation of cell cycle-related proteins by eugenol and/or 1,25(OH)$_2$ vit D$_3$. The HL-60 cells were treated for 3 days with eugenol (150 μM) or 1,25(OH)$_2$ vit D$_3$ (3 nM) alone or in combination with both drugs, and then lysed in RIPA buffer. Equal amount of each sample was loaded onto a 12% SDS-polyacrylamide gel. The separated proteins were analyzed by Western blotting using specific antibodies against cdk2, cdk4, cyclin A, and p27.

accumulation in the G_0/G_1 phase, although the eugenol alone did not affect the cell cycle progression (Fig. 4). We also examined whether the expression of cell cycle proteins related to G_0/G_1 arrest is regulated by eugenol. As shown in figure 5, the combined treatment with eugenol and low dose of 1,25(OH)$_2$ vit D$_3$ more decreased the cdk2, cdk4 and cyclin A, and more increased p27 level than treatment with eugenol or 1,25(OH)$_2$ vit D$_3$ alone. These results indicates that the eugenol potentiates G_0/G_1 arrest of the HL-60 cells by cooperative acting with 1,25(OH)$_2$ vit D$_3$ on regulation of the cell cycle protein expressions.

Enhancement of monocytic differentiation by combined treatment with eugenol and 1,25(OH)$_2$ vit D$_3$

High concentration of 1,25(OH)$_2$ vit D$_3$ (100 nM) can mediate monocytic differentiation of the HL-60 cells by inducing cell arrest at G_0/G_1 phase. To investigate whether the additive activities of eugenol and 1,25(OH)$_2$ vit D$_3$ have an effect on cell differentiation, the HL-60 cells were cotreated with 150 μM of eugenol and suboptimal concentration of 1,25(OH)$_2$ vit D$_3$ (3 nM), which can not induce effectively the differentiation of HL-60 cells. After incubation for four days, the expression of CD14 surface antigen, a differentiation marker for monocytes/macrophages, was detected by flow cytometric analysis. Only 4% or 49% of the cells treated with eugenol (150 μM) or 1,25(OH)$_2$ vit D$_3$ (3 nM) alone, respectively, were positively stained by the FITC-conjugated monoclonal antibody against human CD14. However, the CD14 expression was more increased (64%) when the cells were treated with the combination of eugenol and 1,25(OH)$_2$ vit D$_3$ (Fig. 6). These results suggest that eugenol promotes 1,25(OH)$_2$ vit D$_3$-triggered monocytic differentiation of HL-60 cells.

Differentiation therapy is a potential alternative approach to cytotoxic chemotherapy, because immature cancerous cells can be induced non-replicating mature cells without damages to the normal cells. ATRA treatment for APL is an example of the effective differentiation therapy in clinic [9,10,12]. 1,25(OH)$_2$ vit D$_3$ is also to be noticeable as a differentiation-inducing agent and has been actively studied [1,8]. However, the clinical trials of 1,25(OH)$_2$ vit D$_3$ have been limited due to its hypercalcemic side effect. To overcome the limitation, combined treatments have been tried with a low dose of 1,25(OH)$_2$ vit D$_3$ and another drugs which potentiate the differentiation activity of 1,25(OH)$_2$ vit D$_3$ [14,15]. In the present study, we demonstrated that, for the first time, a combination with a low dose of 1,25(OH)$_2$ vit D$_3$ (3 nM) and 150 μM of eugenol strongly induced the monocytic differentiation of HL-60 cells, whereas the each compound induces a little or no cell differentiation as an individual treatment. Our findings suggest that the ability of eugenol to promote differ-

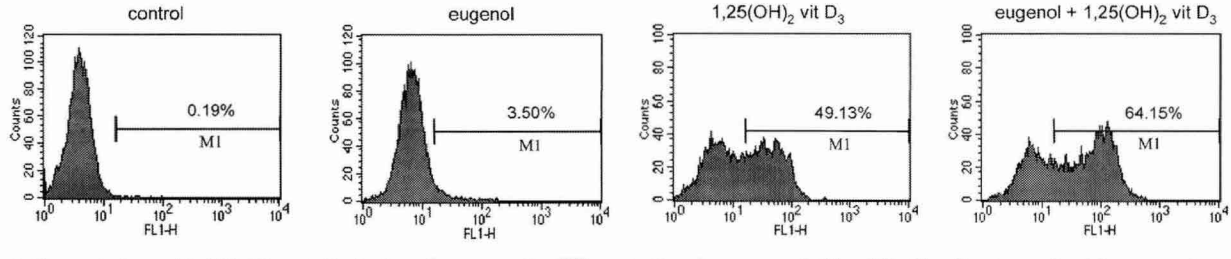


Fig. 6. Potentiation of 1,25(OH)$_2$ vit D$_3$-induced monocytic differentiation by eugenol. The HL-60 cells, treated with eugenol and/or 1,25(OH)$_2$ vit D$_3$ for 3 days, were washed twice with buffer A (PBS, 0.1% sodium azide, 1% heat-inactivated fetal bovine serum). After being resuspended in buffer A, cells (1 x 10^6 cells) were incubated with FITC-conjugated monoclonal anti-human CD14 antibody on ice for 30 min, and then analysed by a flow cytometer. Control represents untreated cells.

entiation might be applicable to an effective therapeutic strategy for treating APL.

Acknowledgment

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea. (A050209)

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초록 : Eugenol과 1α,25-dihydroxyvitamin D_3의 병합처리에 의한 HL-60 세포의 분화 유도

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Eugenol은 여러가지 향신료에 있는 필수 오일의 주요 성분으로서 악성 종양 세포의 성장을 저해하고 사멸을 유도한다고 보고되었다. 본 연구에서는 eugenol이 세포 분화 유도에 관여하는지를 조사하기 위하여 HL-60 전골수성 백혈병 세포의 분화에 미치는 eugenol의 효과를 연구하였다. HL-60 세포에 eugenol (150 μM)을 가했을 때 세포성장이 저해되었으며 1,25$(OH)_2$ vit D_3 (3 nM)와 병합처리시에는 더 큰 저해효과를 보였다. 이 때, eugenol은 1,25$(OH)_2$ vit D_3에 의해 유도되는 세포주기의 G_0/G_1단계 정지를 더욱 증가시킴을 알 수 있었다. 또한, eugenol과 1,25$(OH)_2$ vit D_3를 병합처리 하였을 때에는 G_0/G_1단계의 정지와 관련된 세포주기 조절인자인 p27 level를 상호협동적으로 증가시켰을 뿐만 아니라 cyclin A, cdk2, cdk4 level를 감소시켰다. 또한 유세포분석실험을 통하여, CD14 (단핵세포 표지 인자)의 발현이 eugenol과 1,25$(OH)_2$ vit D_3를 병합처리한 세포에서 단독처리시보다 더 증가함을 알 수 있었다. 이러한 결과들은 eugenol이 1,25$(OH)_2$ vit D_3와 상호협동적으로 작용하여 저농도의 1,25$(OH)_2$ vit D_3에 의해 자극된 세포 분화 신호를 더욱 더 증대 시킴을 나타낸다. 이러한 eugenol에 의한 세포 분화 유도 작용은 암의 화학적예방 효과에 유용하게 응용될 수 있을 것으로 기대된다.