Arch Pharm Res Vol 25, No 4, 480-484, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Costunolide Induces Differentiation of Human Leukemia HL-60 Cells

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(Received May 22, 2002)

Costunolide has been reported to be a cytotoxic and chemopreventive agent. This work investigated the mechanism of the antiproliferative effect of costunolide and determined that it induced differentiation of the human leukemia cell line HL-60. Costunolide exhibited a potent antiproliferative activity against HL-60 cells. It was also found to be a potent inducer of differentiation in human leukemia derived HL-60 cells through the examination of differentiation markers, as assessed by the reduction of nitroblue tetrazolium, the increase in esterase activities and phagocytic activity, morphology change and the expression of CD14 and CD66b surface antigens. These results, accompanied by a decline in the expression of c-myc protein, suggest that costunolide induces differentiation of human leukemia cells to granulocytes and monocytes/macrophages lineage.

Key words: Costunolide, Differentiation, Granulocyte, Monocyte, HL-60 cell

INTRODUCTION

Costunolide, one of numerous germacranolides, belongs to the group of sesquiterpene lactones obtainable from natural origins (Fig. 1). The biological activities of costunolide have been reported to have cytotoxic (Yamahara *et al.*, 1985), anti-ulcer (Yoshikawa *et al.*, 1993), antiviral (Chen *et al.*, 1995) and chemopreventive effects (Mori *et al.*, 1994; Ohnishi *et al.*, 1997). As one of the immunological aspects of costunolide, it inhibits the killing activity of cytotoxic T lymphocytes by preventing the increase in tyrosine phosphorylation in response to the crosslinking of T-cell receptors (Taniguchi *et al.*, 1995).

Leukemia is mostly a disease with self-growing white blood cells, which are histologically and functionally immature. A possible approach to the treatment of acute leukemia patients is to induce differentiation or to inhibit clonal proliferation of the leukemic cells. Attention has focused on the human promyelocytic HL-60 leukemia cell line (Collins *et al.*, 1978), since HL-60 provides a useful *in vitro* model system for studying the cellular and molecular events involved in the differentiation process. Any inducer of HL-60 differentiation is commonly accepted to have potentially therapeutic importance. Terminal differentiation of HL-60 cells can be monitored by changes of morphological, biochemical, and immunological properties. The differentiated HL-60 phenotype is characterized by growth inhibition, increased adherence, loss of cell-surface transferrin receptors, increased monocyte surface markers, induction of α-naphthyl acetate (nonspecific) esterase and certain patterns of protein phosphorylation (Yam *et al.*, 1971). Certain compounds such as 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), which is known to be efficacious cancer preventative agents, are potent inducers of HL-60 cell differentiation (Tanaka *et al.*, 1983;

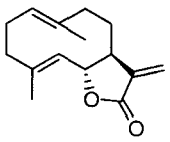

Fig. 1. Chemical structure of costunolide

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Koeffler *et al.*, 1984; Ostrem *et al.*, 1987; Zhou *et al.*, 1989), and appear to be clinically effective against myeloproliferative disorders. Differentiation inducers allow cell differentiation to continue, whereas the decrease of cellular proliferation by differentiation inducers results primarily from maturation related growth arrest (Reiss *et al.*, 1986). Furthermore, the cell differentiation inducer itself has cytotoxic effects on cancer cells. In this study, the cytotoxic effect of costunolide was assessed by its differentiation activity against HL-60 cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), nitroblue-tetrazolium (NBT), $1\alpha,25(OH)_2D_3$, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), α-naphthyl acetate esterase kit and 3-hydroxy-2-naphthoic acid *O*-toluidine (naphthol AS-D chloroacetate) esterase kit from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein-isothiocyanate (FITC)-labeled anti-human CD14 mouse monoclonal antibody and anti-human CD66b mouse monoclonal antibody were purchased from PharMingen Co. (San Diego, CA, USA), anti-human c-myc mouse monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Costunolide was isolated from the stem bark of Magnolia sieboldii, as previously reported (Park et al., 1996).

Growth-inhibition Assay

Cells were seeded at a concentration of 2×10^5 cells/ml, maintained for logarithmic growth by passaging them every 2 to 3 days, and incubated for 2 to 4 days with costunolide at various concentrations. The cell viability was checked by the trypan blue exclusion method. The percentage of inhibition was calculated as:

$100 - T/C \times 100$,

where T and C were the number of cells in treated and control cultures, respectively.

Differentiation Assay

1) NBT reduction test: Cells were incubated with NBT (1.0 mg/ml) at 37°C for 30 min, and the percentage of HL-60 cells capable of reducing NBT was determined by counting the number of cells which contained precipitated formazan particles. TPA was used as a stimulator for the formation of formazan. 2) Phagocytosis test: HL-60 cells (1×10^6 cells/ml) were suspended in serum-free RPMI 1640 medium containing 0.2% latex particles (average diameter, 0.81 μm; Sigma Chemical) and incubated at 37°C for 4 h. After incubation, the cells were washed once with phosphate-buffered saline (PBS). The cells containing more than ten latex particles were counted as phagocytic cells. 3) Esterase activity test: A smear preparation was chemically stained for α-naphthyl acetate esterase (Ben-Baruch *et al.*, 1994) and naphthol AS-D chloroacetate esterase (Zhang *et al.*, 1992) by standard techniques. 4) Flow cytometry test: HL-60 cells exposed to costunolide were collected, washed twice with ice-cold PBS, incubated with FITC-labeled anti-CD14 or anti-CD66b antibody on ice for 30 min, and washed twice with PBS. Antibody binding to cells was quantified using a FACS flow cytometer (Becton Dickinson Co., Germany).

Western blot analysis

Cellular proteins were extracted from control and costunolide treated HL-60 cells. The washed cell pellets were resuspended in ELB buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin, and incubated for 15 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein was determined by Bio-Rad (CA, U.S.A.) protein assay reagent as described by the manufacturer. Cellular protein (100 μg) from treated and untreated cell extracts was electroblotted onto nitrocellulose membrane following electrophoretic separation on a 10% SDS-polyacrylamide. The immunoblot was incubated overnight with blocking solution (5% skim milk in 0.02% Tween 20 containing PBS) at 40°C, followed by incubation for 4 h with a 1:500 dilution of anti-c-myc antibody. Blots were washed 2 times with PBS, incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed again three times with PBS, and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RESULTS

The effect of costunolide on the proliferation of HL-60 cells was examined. Costunolide showed antiproliferative activity, because the cell growth of HL-60 cells was inhibited in a concentration-dependent manner (Fig. 2). The cell viability, assessed by trypan-blue dye exclusion, was not increased even at high concentrations of costunolide. At a concentration of 1 μM costunolide, the inhibition of cell growth became apparent 3 days after exposure and reached around 50% growth inhibition one

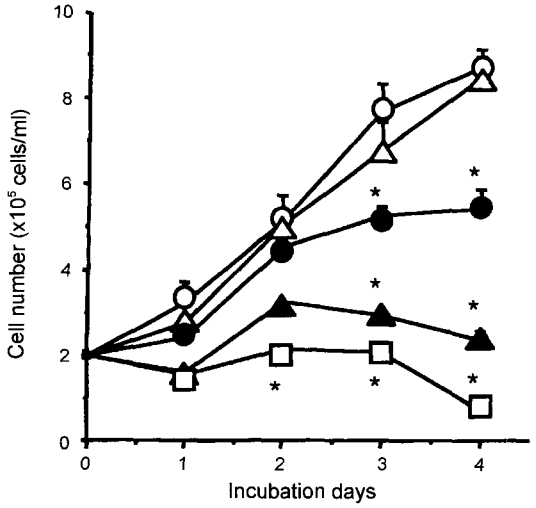

Fig. 2. Effect of costunolide on the growth of HL-60 cells. Cells were treated with increasing concentrations of costunolide for 4 days. Each point with a bar represents the mean ± S.D. of three independent experiments. *Significantly different from the control at $P < 0.01$. ○: control; △: 0.5 μM ●:1 μM, ▲ : 1.5 μM, □: 2 μM.

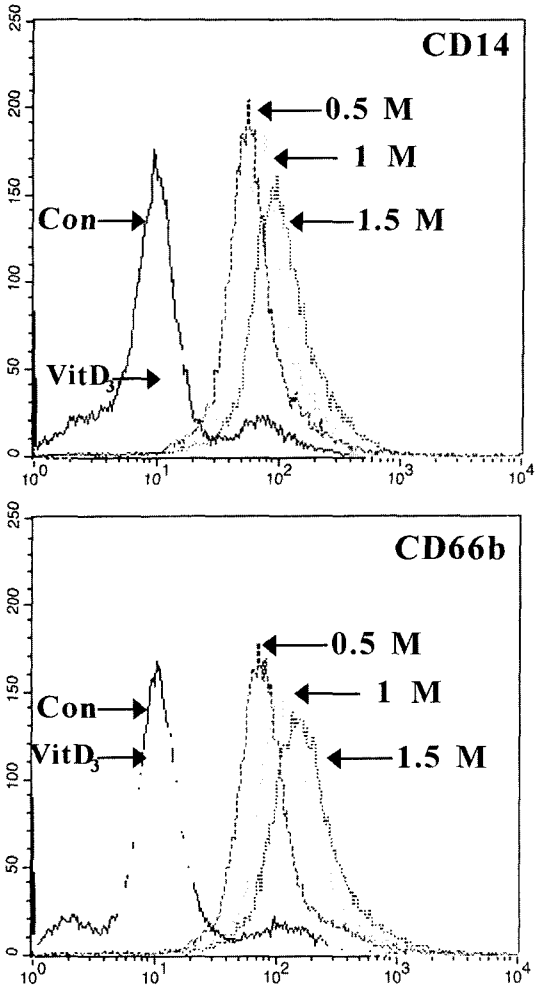

Fig. 3. FACS analysis of the expression of CD14 and CD66b antigens on HL-60 cells treated with either costunolide or 1α,25(OH)$_2$D$_3$.

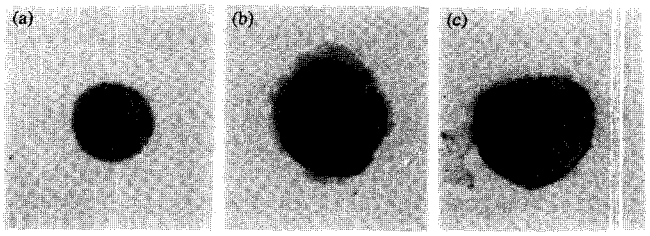

Fig. 4. Morphologic analysis of HL-60 Cells. Cells were treated with or without costunolide for 4 days, fixed and stained with May-Grunwald Giemsa, x 800. (a) untreated control, (b) treated with 1 μM costunolide, (c) treated with 0.02 μM 1α,25(OH)$_2$D$_3$.

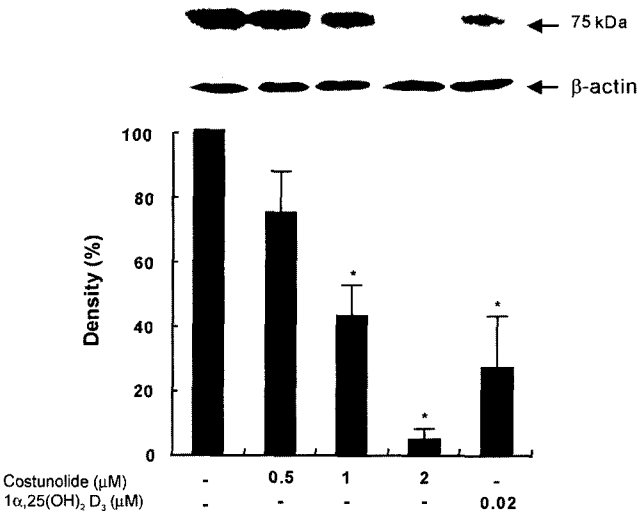

Fig. 5. Levels of c-myc protein in costunolide-treated HL-60 cells. Cellular protein (100 μg) from treated and untreated cell extract was analyzed by Western blotting using anti c-myc antibody as described in the Materials and Methods section. Data represent the mean ± S.D. from three separate cell preparations. *Significantly different from the untreated control at $P < 0.01$.

day later (Fig. 2).

To examine whether the partial inhibition of HL-60 growth by costunolide was associated with terminal differentiation, we performed the NBT test for the detection of granulocytes and phagocytosis for macrophage-like cells. When HL-60 cells were incubated with costunolide at a concentration of 1 μM for 4 days, approximately 80% of the cells became stainable with NBT, compared to only 13.2% of the untreated cells (Table 1). 1α,25 (OH)$_2$D$_3$ (20 nM), the potent inducer of HL-60 cell differentiation, produced an NBT-reproducible cell rate of 58.4%. Treatment of HL-60 cells with 1 μM of costunolide for 4 days resulted in 75% increase of the α-naphthyl acetate esterase activity, but the effect of costunolide and 1α,25 (OH)$_2$D$_3$ on the naphthyl AS-D chloroacetate esterase activity was relatively mild. Cells treated with these compounds showed apparent phagocytic activity. Moreover, as shown in Fig. 3, 0.5 to

1.5 μM costunolide significantly increased the expression of both membrane antigens CD14 and CD66b, whereas 1α,25$(OH)_2D_3$ (20 nM) only mildly increased the expression of both CD14 and CD66b.

Morphological evaluation of the cells after May-Grunwald Giemsa stain indicated that the costunolide-treated cells became larger than the untreated cells and developed a more granulated appearance, developments characteristic of differentiated cells such as granulocytes and monocytes/macrophages (Fig. 4).

It was examined whether the costunolide-related growth inhibition induced by differentiation was accompanied by modulation of the growth-related oncoprotein c-myc. After treatment with costunolide for 4 days, immunoblot analysis for c-myc protein in the cells was performed (Fig. 5). Costunolide reduced the c-myc protein level in a concentration-dependent manner.

DISCUSSION

Leukemia cells are blocked at some stages of the maturation processes and display a high proliferative capacity. The potential value of differentiation inducers as therapeutic agents resides in their ability to overcome the mature blockade. The present study has demonstrated that costunolide exerts a potent differentiation-inducing activity on promyelocytic leukemia HL-60 cells. Costunolide produced changes in the morphology and expression of markers of differentiation in HL-60 cells, such as NBT reducing ability, increased phagocytotic activity and the appearance of esterase activities. Moreover, this compound markedly increased the expression of both cell surface antigens CD14 and CD66b, which were reported as marker antigens of monocytes and granulocytes, respectively (Eades-Perner *et al.*, 1998), whereas 1α,25$(OH)_2D_3$ enhanced the expression of CD14 more than CD66b. These results indicate that costunolide is a new, potent inducer of differentiation in HL-60 human leukemia cells to granulocytes and monocytes/ macrophages.

Although the mechanism of costunolide's differentiation induction is not clear, it appears that c-myc plays an important role in the modulation of the compound's cell differentiation activity. We observed that this compound decreased c-myc protein expression. The c-myc gene product is a nuclear protein and has been implicated in the control of normal cell growth, as well as transformation, but its exact function is unknown. A decrease in c-myc mRNA has been demonstrated *in vitro* during chemically induced differentiation of various cell lines and this decline was either gradual or associated with the biphasic disappearance of the c-myc mRNA during differentiation of the leukemia cell lines (Filmus and Buick, 1985; Tadokaro and Ikawa, 1986). On the other hand, it has been reported that suramin-induced down-regulation of c-myc in HL-60 cells is not sufficient to trigger differentiation toward granulocytes or monocytes (Ben-Baruch *et al.*, 1994). These reports suggest that c-myc expression plays a central role in determining whether these leukemia cells remain in a continuous cycle of growth and proliferation or enter a differentiation pathway. In similar ways this modulation of c-myc protein expression probably accounts for the anti-proliferative and differentiation-inducing effect of costunolide in HL-60 cells. Our ongoing study showed that the antiproliferative activity of costunolide was caused via premature activation of cdc2 based on the following evidence: (1) G_1-synchronized cells, which are not ready for mitosis, were more sensitive to costunolide, and (2) cdc2 mRNA was transcriptionally activated after costunolide stimulation.

Costunolide is a sesquiterpene lactone that has been reported to significantly inhibit tyrosine phosphorylation induced by crosslinking of the CD3 molecule (Taniguchi *et al.*, 1995). These observations suggest that if the site of the action of costunolide is limited to tyrosine kinase or a closely associated reaction, then the hypothetical common step in differentiation is likely to be related to tyrosine residues in cellular proteins. In this respect, it may be worth mentioning that genistein and herbimycin A, inhibitors of protein phosphorylation activity of protein tyrosine kinase *in vitro*, also induce differentiation of several cells under certain condition (Kondo *et al.*, 1989). Also of interest is the fact that costunolide has an electrophilic function of exomethylene as in the known differentiation inducer of 1α,25$(OH)_2D_3$, although costunolide has α-methylene-γ-lactone whereas 1α,25$(OH)_2D_3$ has a partial structure with conjugated triene including exomethylene. However, both chemicals share reactive exomethylenes, and it has been suggested that this feature could be the explanation for costunolide following the differentiation phenomena of 1α,25$(OH)_2D_3$. Nevertheless, costunolide may show other differentiation features dissimilar to those of 1α,25$(OH)_2D_3$ regarding the selectivity, potency and further toxicity *in vivo* in the bioactivity because both are obviously different in their entire structures. On the other hand, it has been reported that flavonoids exhibiting the inhibitory activity of protein tyrosine kinase also induced apoptosis (Iwashita *et al.*, 2000). Recently, we showed that costunolide induced apoptosis in HL-60 cells by its reactive oxygen species-mediated, mitochondrial membrane potential loss (Lee *et al.*, 2001), but at concentrations (above 5 μM) much higher than required for differentiation activity.

In summary, the induction of HL-60 cell maturation produced by costunolide introduces its feasibility as a therapeutic approach to the treatment of leukemia that involves terminal differentiation.

ACKNOWLEDGEMENTS

Financial support of a grant (HMP-00-B-21600-0124) from the 2000 Good Health R&D Project, the Ministry of Health & Welfare, Korea is gratefully acknowledged.

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