Evaluation of anticancer activity of dehydrocostuslactone *in vitro*

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Abstract. Dehydrocostuslactone is a sesquiterpene lactone derived from *Saussurea lappa*, a plant used in traditional herbal medicines. The anti-proliferative activity of dehydrocostuslactone was investigated in human breast cancer (MDA-MB-231, MDA-MB-453 and SK-BR-3) and ovarian cancer (SK-OV-3 and OVCAR3) cell lines using the methyl thiazolyl tetrazolium assay. In the cells, exposure to dehydrocostuslactone resulted in a dose-dependent decline in cell proliferation. The IC_{50} value was found to be 21.5, 43.2, 25.6, 15.9 and 10.8 μM in MDA-MB-231, MDA-MB-453, SK-BR-3, SK-OV-3 and OVCAR3 cells, respectively. Dehydrocostuslactone exerted its antiproliferative effects by inducing cell cycle arrest and apoptosis. Cell cycle distribution and apoptosis were analyzed using flow cytometry in cell lines exposed to 10 μM dehydrocostuslactone for 48 h. Compared to the controls, exposure to dehydrocostuslactone resulted in accumulation in the G2/M phase and a marked increase in the apoptotic cell population. These results suggest that dehydrocostuslactone has potential anticancer properties.

Introduction

Breast and ovarian cancer continue to be major health threats for women. Breast cancer is the most frequently occurring cancer in women, and an increased familial risk of breast cancer is associated with an increased risk of ovarian cancer (1,2). A number of studies have examined the use of phytochemicals to prevent or treat cancer, since plant products are considered valuable sources of novel anticancer drugs.

Dehydrocostuslactone is one of the main sesquiterpene lactones found in *Saussurea lappa*, a traditional herbal medicine that has been used to treat cancer in India and Japan (3). *Saussurea lappa* has several biological effects, including anti-inflammatory activity (4-6). It has also been reported that *Saussurea lappa* induces G2 arrest in human cancer cells, which may be correlated with the induction of apoptosis (7,8). The molecules responsible for the therapeutic activity of *Saussurea lappa* have not been identified. We therefore evaluated the anticancer activity of dehydrocostuslactone in three human breast cancer cell lines, MDA-MB-231, MDA-MB-453 and SK-BR-3, and two ovarian cancer cell lines, OVCAR3 and SK-OV-3, to identify a novel molecule candidate.

Materials and methods

Cell culture and dehydrocostuslactone treatment. Human breast carcinoma MDA-MB-231, MDA-MB-453 and SK-BR-3 cells and ovarian carcinoma OVCAR3 and SK-OV-3 cells were purchased from the Korean Cell Line Bank. Each cell line was routinely maintained in RPMI-1640 or DMEM (Invitrogen Molecular Probes, Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin (Gibco) at 37˚C in a humidified atmosphere containing 5% CO_2. For the cell proliferation assay, the cell lines were exposed to either dehydrocostuslactone (1-100 μM) or vehicle alone for 48 h. For the cell cycle and apoptosis assay, cells were exposed to dehydrocostuslactone (10 μM) or vehicle alone for 48 h. Dehydrocostuslactone was purchased from Wako Pure Chemical Industries, Ltd. (Japan) and dissolved in DMSO at a final concentration of 0.1% in medium.

Cell proliferation assay. The inhibitory effect of dehydrocostuslactone on cell proliferation was determined by the methyl thiazolyl tetrazolium (MTT) assay. Cells were treated with dehydrocostuslactone concentrations ranging from 1 to 100 μg/ml. After 48 h of incubation, MTT was added. Four hours later, DMSO was added to each well to dissolve the resulting formazan crystals, and then absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices). The IC_{50} value (concentration of extract required to inhibit cancer cell proliferation by 50% of control levels) was estimated from the plot. The breast and ovarian carcinoma cell lines were respectively treated with DMSO compound solvent alone for use as the controls.

Cell cycle analysis. Cells were harvested, washed with cold PBS and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20˚C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed twice with cold PBS. RNase A (20 μg/ml final concentration) and propidium iodide staining solution (50 μg/ml final concentration) were added, then the cells were incubated for 30 min at 37˚C in the dark. The cells were analyzed using a

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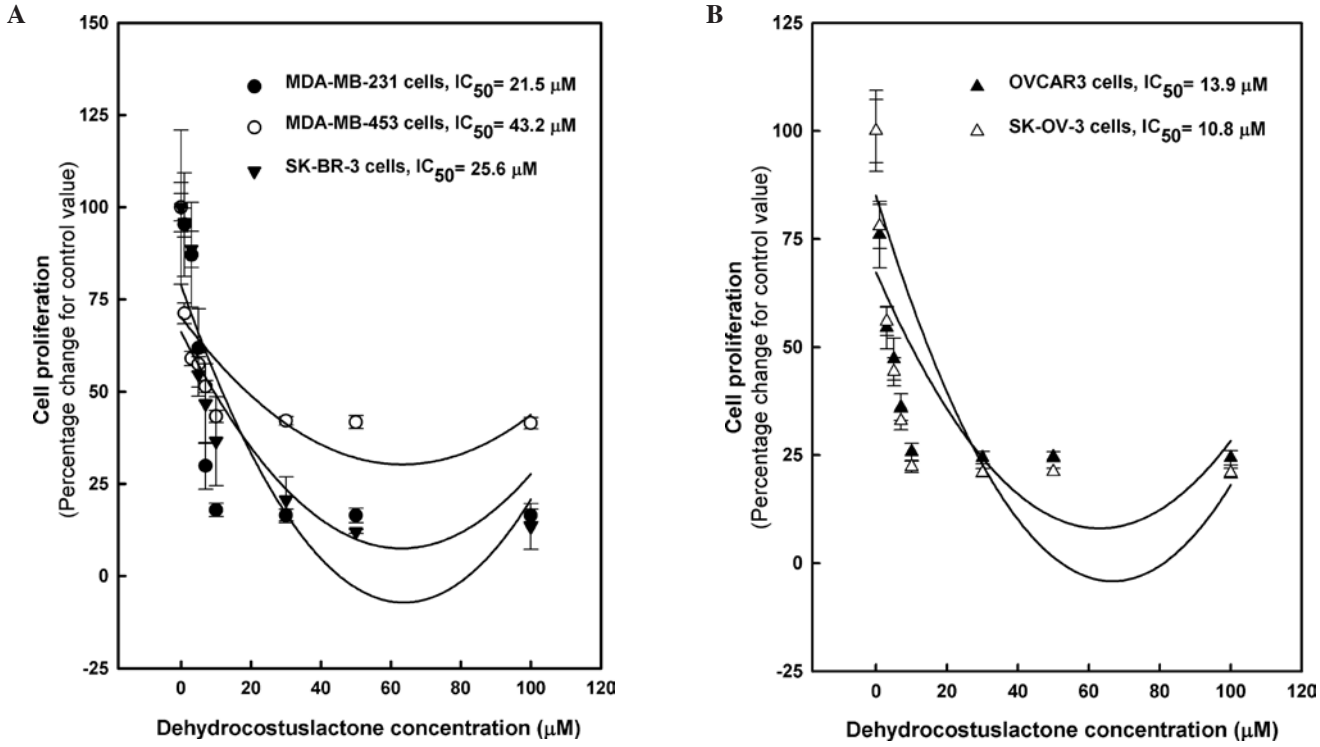

Figure 1. Effect of dehydrocostuslactone on cell proliferation in human breast (A) and ovarian (B) cancer cells. Each cell line was exposed to dehydrocostuslactone at various concentrations (1, 3, 5, 7, 10, 30, 50 and 100 μM) for 48 h. Data are expressed as the mean ± SD of four independent experiments. Reductions in cell density were plotted against various concentrations of dehydrocostuslactone (1-100 μM), and the IC_{50} value (concentration of extract required to inhibit cancer cell proliferation by 50% of control levels) was estimated from the plot. The cell lines were respectively treated with DMSO compound solvent alone for use as the controls.

FACSCalibur instrument (BD Biosciences Clontech, San Jose, CA, USA) equipped with CellQuest 3.3 software. ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in various phases of the cell cycle.

Annexin V assay. An Annexin V-FITC apoptosis kit (BD ApoAlert™, BD Biosciences) was used for apoptosis detection. Cells were trypsinized, washed twice in ice-cold PBS and resuspended in 500 μl binding buffer (Sigma). Annexin V and propidium iodide solution were added to the cell preparations and cells were incubated for 25 min in the dark. Binding buffer (400 μl) was then added to each tube and the samples were analyzed by a FACSCalibur instrument equipped with CellQuest 3.3 software.

Statistical analysis. Data were expressed as the percentage compared with vehicle-treated control cells, which were arbitrarily assigned a value of 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $P<0.05$.

Results

Antiproliferative effect of dehydrocostuslactone. The effect of dehydrocostuslactone on cell proliferation was measured using the MTT assay in human ovarian cancer (SK-OV-3 and OVCAR3) and breast cancer (MDA-MB-231, MDA-MB-453 and SK-BR-3) cells exposed to dehydrocostuslactone at various concentrations (1, 3, 5, 7, 10, 30, 50 and 100 μM) for 48 h. In the cell lines studied, dehydrocostuslactone significantly decreased cell proliferation in a dose-dependent manner ($P<0.05$) (Fig. 1). As shown in Fig. 1, the ovarian cancer cells were more sensitive than the breast cancer cells in terms of cell growth. Statistical differences in cell proliferation were first noted as the inhibition of cell proliferation in ovarian and breast cancer cells exposed to 1 and 5 μM of dehydrocostuslactone, respectively. In addition, dehydrocostuslactone exhibited antiproliferative effects against MDA-MB-231, MDA-MB-453, SK-BR-3, SK-OV-3 and OVCAR3 cells with IC_{50} values of 21.5, 43.2, 25.6, 15.9 and 10.8 μM, respectively.

Cell cycle arrest by dehydrocostuslactone. To verify the anticancer activity of dehydrocostuslactone, the cell lines were exposed to dehydrocostuslactone at 10 μM for 48 h, and then the cell cycle was analyzed using flow cytometry (Fig. 2). Compared to the control cells, exposure to dehydrocostuslactone significantly increased the cell population in the G2/M phase by 2.3-, 1.3- and 2.5-fold in the human breast cancer cell lines MDA-MB-231, MDA-MB-453 and SK-BR-3, respectively. A similar pattern of cell cycle arrest at the G2/M phase and apoptosis was also observed in the ovarian cancer cell lines (2.6 and 2.5-fold increase, OVCAR3 and SK-OV-3 cells, respectively). The accumulation of the cell population in the G2/M phase was accompanied by a concomitant increase in the cell population in the S phase; an increase of 1.5-, 1.6-, 1.6-, 2.3- and 3.0-fold was observed for MDA-MB-231, MDA-MB-453, SK-BR-3, OVCAR3 and SK-OV-3 cells, respectively.

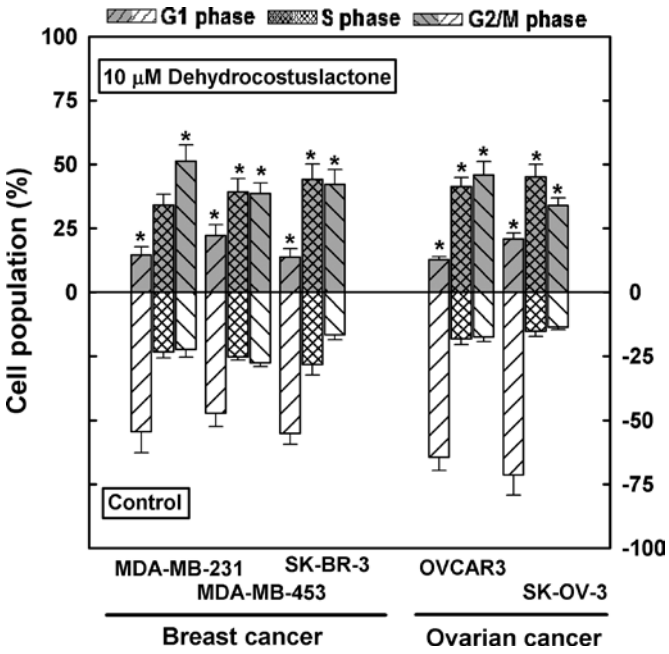

Figure 2. Effect of dehydrocoslactone on cell cycle distribution in human breast and ovarian cancer cells. Each cell line was exposed to 10 μM dehydrocostuslactone (lowest concentration to elicit a response) for 48 h. Data are expressed as the mean ± SD of four independent experiments. Values are expressed as the percentage of the cell population in the G1, S and G1/M phase of the cell cycle. *P<0.05, significantly different from the respective control level (0.1% DMSO in medium; i.e, a dehydrocostuslactone concentration of 0).

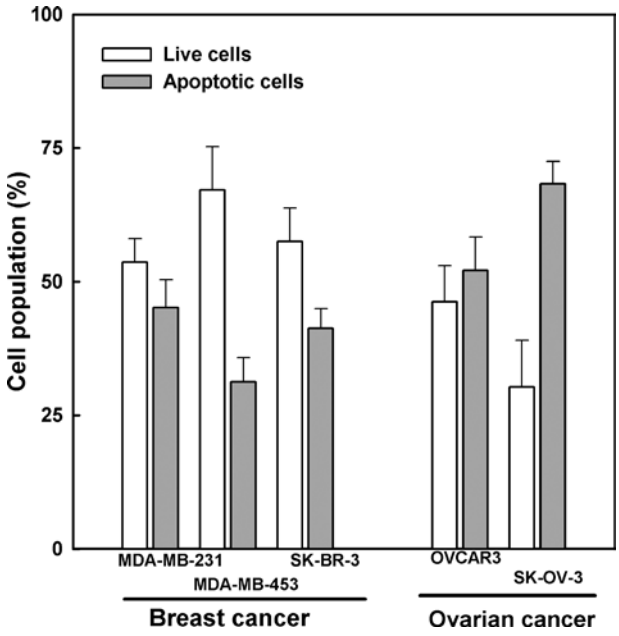

Figure 3. Apoptosis induction by dehydrocoslactone in human breast and ovarian cancer cells. Each cell line was exposed to dehydrocostuslactone at 10 μM (lowest concentration to elicit a response) for 48 h. Data are expressed as the mean ± SD of four independent experiments. The apoptotic cell population in the respective control cells (0.1% DMSO in medium; i.e, a dehydrocostuslactone concentration of 0) was <1% (data not shown).

Pro-apoptotic effects of dehydrocostuslactone. Since dehydrocostuslactone was found to be capable of altering the cell cycle and inhibiting cell proliferation, we next assessed the induction of apoptosis by Annexin/PI staining under the same experimental conditions. In the control cells, the live cell populations exceeded 99.2%, and few apoptotic cells were detected (data not shown). Compared to the controls, the apoptotic cell population of ovarian cancer cells was increased by >50% (52.1 and 68.3% in SK-OV-3 and OVCAR3, respectively), while the apoptotic cell population of the breast cancer cells was significantly increased by 30-45% (Fig. 3).

Discussion

This study was conducted to investigate the potential of dehydrocostuslactone as a novel anticancer candidate for the treatment of female breast and ovarian cancers. Dehydrocostuslactone is the main sesquiterpene commonly found in *Saussurea lappa*, a well-known traditional herbal medicine used for cancer treatment in Asia; however, studies have not been conducted to investigate the anticancer activity of dehydrocostuslactone.

To evaluate the anticancer activity of dehydrocostuslactone, we first observed cell proliferation in various human cancer cell lines exposed to dehydrocostuslactone for 48 h at concentrations ranging from 1 to 100 μM. Dehydrocostuslactone exhibited significant dose-dependent antiproliferative activity. To date, most positive results have been obtained in studies using human ovarian cancer cell lines. The antiproliferative effect of dehydrocostuslactone peaked at 10-30 μM. The ovarian cancer cells were more sensitive to dehydrocostuslactone that the breast cancer cells. Dehydrocostuslactone exhibited active antiproliferative effects against ovarian cancer OVCAR3 and SK-OV-3 cells, with IC_{50} values of less than 15 μM. This is an encouraging result, since ovarian cancer is particularly chemosensitive (9,10). Although surgery is important in the initial therapy of patients with breast or ovarian cancer, most patients require chemotherapy to eliminate any residual microscopic or macroscopic peritoneal implants. Human ovarian cancer cell lines appear to be sensitive to dehydrocostuslactone, suggesting it has anticancer activity (11-13).

We also found that dehydrocostuslactone exerted anti-proliferative effects in several breast cancer cell lines by altering the cell cycle. In cells exposed to dehydrocostuslactone, G2/M phase arrest was induced. In ovarian cancer cells, treatment with dehydrocostuslactone resulted in accumulation in the G2/M phase to a similar degree (~2.6-fold) as that observed in the respective controls. A similar pattern was observed for breast cancer cells, although the effect was weaker than that observed in the ovarian cancer cells (~1.3 to 2.3-fold increase compared to the respective controls). Dehydrocostuslactone has been shown to induce cell cycle arrest at the G2/M phase in several cancer cell lines *in vitro* (14,15). This is consistent with our results, which indicated that exposure to dehydrocostuslactone induces significant cell cycle arrest.

In this study, the inhibitory effect of dehydrocostuslactone on cancer cell proliferation resulted in cell cycle arrest and apoptosis. Under the same conditions, exposure to dehydrocostuslactone resulted in significant apoptosis in all the cell lines examined. This is consistent with reports that dehydrocostuslactone is an effective anticancer agent

capable of inducing apoptosis (14-17). Apoptosis is an important series of events that lead to programmed cell death, and is essential for development and tissue homeostasis. The potential mechanisms underlying the apoptotic process involve factors regulating the balance between the induction and inhibition of apoptosis. Recently, the regulation of apoptosis has been proposed as a promising target for cancer chemotherapy (18-21). Thus, apoptosis induced by dehydrocostuslactone may have a significant potential anticancer effect.

Taken together, the results of this study suggest that dehydrocostuslactone possesses significant antiproliferative activity via cell cycle arrest and apoptosis, particularly in ovarian cancer. Due to these characteristics, dehydrocostuslactone may serve as a useful anticancer drug. However, further studies are needed to elucidate its exact mechanisms of action.

Acknowledgements

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