Apoptosis Induced in Human Cell Lines by a Butanol Extract from *Chlorophytum comosum* Roots

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The antiproliferative effects of a *n*-butanol extract from *Chlorophytum comosum* (*C. comosum*) (spider plant) roots was tested *in vitro* against four human cell lines: HeLa, CCRF-HSB-2, HL-60 and U937 cells. The extract was found to have cell antiproliferative effects against HeLa, CCRF-HSB-2, HL-60 and U937 cell lines, with an IC_{50} value of 6.6, 5.4, 8.7 and 10.2 μg/ml, respectively. The dying cells showed characteristics of apoptosis, such as DNA fragmentation, as assessed by DNA electrophoresis analysis and the Terminal deoxynucleotidyl transferase (TdT)-mediated biotin dUTP Nick End-Labeling (TUNEL) method. These results indicate that extract from *C. comosum* roots can induce apoptosis in human cell lines.

Key words —— *Chlorophytum comosum*, apoptosis, CCRF-HSB-2

INTRODUCTION

Apoptosis is widely observed in different kind of cells in most organisms, from nematodes to mammals, and it is generally accepted that it plays an important role in physiological processes during maturation of the immune system, embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover.[1] Furthermore, apoptosis is induced in various cell lines *in vitro* by incubating the cells with chemicals such as antitumor agents.[2] Apoptosis is a unique morphological type of cell death that is characterized by chromatin condensation, membrane blebbing, and cell fragmentation.[3–5] The most prominent event in the early stages of apoptosis is internucleosomal DNA cleavage by undefined endonuclease activities.[6–8] We previously reported that the human leukemia cell line CCRF-HSB-2 undergoes apoptosis and cell-cycle arrest in response to DNA damage caused by various antitumor agents, and proposed that this cell line would be useful in screening compounds during the development of new antitumor agents.[9]

Apoptosis, the intrinsic death program of a cell, plays a crucial role in the regulation of tissue homeostasis. Many studies have indicated that an imbalance between cell death and cell proliferation may result in tumor formation. In addition, the killing of tumor cells by diverse cytotoxic approaches, such as anticancer drugs, gamma-irradiation or immunotherapy, is predominantly mediated through the induction of apoptosis. Apoptotic therapy has attracted many groups of investigators, and many companies have entered the race to develop the first generation of apoptotic anticancer medications.[10]

Chlorophytum comosum (*C. comosum*), a popular houseplant in Japan, is a grass-like, clump-forming, evergreen perennial of the lily family. *C. comosum* originated from South Africa and is used as a traditional folk medicine in China for treating bronchitis, fractures and burns.[11] Mimaki *et al*. have shown that *C. comosum* roots have antitumor-promoter activity,[12] there are few reports on the biological behavior of *C. comosum* and its specific components so far. In this paper, we have examined the antiproliferative ability of *C. comosum* to induce apoptosis in several human cell lines in order to find biologically active substances in *C. comosum*.

MATERIALS AND METHODS

Chemicals —— 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Dojin Laboratories (Kumamoto, Japan). Agarose, dimethyl sulfoxide (DMSO) and *n*-butanol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Most reagents used were the purest grade available and

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were purchased from Wako Pure Chemical Industries Ltd.

Cell Lines and Culture Conditions —— The cell lines used in this study, CCRF-HSB-2 (human T cell leukemia cell line), HL-60 (human promyelocytic leukemia cell line), HeLa (human cervical adenocarcinoma) and U937 (human monocyte tumor cell line), were provided by RIKEN Cell Bank (Tsukuba, Japan).

CCRF-HSB-2, HL-60, and HeLa cell lines were cultured in RPMI1640 medium (JRH Biosciences, Lenexa, KS, U.S.A.) supplemented with 10% fetal calf serum (JRH Biosciences). U937 cells were cultured with E-RDF medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum at 37°C in 5% CO_2.

Extraction of *C. comosum* —— *C. comosum* plants were grown in soil in pots at room temperature. Ten grams of roots of *C. comosum* was minced and stirred with 10 ml of water at 4°C for 1 hr. The aqueous solution was extracted with 10 ml of *n*-butanol and the *n*-butanol layer was evaporated to dryness. A residue of the *n*-butanol layer obtained was 40 mg dry weight.

Cell Proliferation Assay —— Four human cell lines, U937, CCRF-HSB-2, HeLa and HL60, were used for cell proliferation assay. Near-confluent cells were collected and diluted to a concentration of 5×10^4 cells/ml with culture medium. One hundred microliter aliquots of cell suspension were dispensed into each well of 96-well plates. The residue from *C. comosum* was redissolved in water and filtered through a 0.22-μm filter (Milex GP, Millipore Co., Bedford, Bedford, U.S.A.). Ten microliters of the redissolved sample solution of root extract diluted with culture medium was added to each cell suspension well to give each test concentration. After 72 hr of incubation at 37°C in 5% CO_2, the cell proliferation were assayed by the WST-1 method.[13] In brief, stock solutions of 10 mM WST-1 and 0.2 mM 1-methoxy PMS were prepared in 20 mM HEPES buffer (pH 7.4), and water, respectively. Equal amounts of WST-1 and 1-methoxy PMS solutions were mixed as a working solution just before analysis. A 100 μl aliquot of cell suspension was dispensed into the 96-well plates, 10 μl of the working solution was added to each well, and the plates were then incubated for an additional 2 hr. The absorbance of each well was measured at 450 nm with the reference wavelength at 655 nm using a microplate reader (Model 550, Nippon Bio-Rad Laboratories, Tokyo, Japan). Percent of cell proliferation was calculated as follows: Cell proliferation (%) = [($A_{450/655}$ of sample – $A_{450/655}$ of blank)/($A_{450/655}$ of control – $A_{450/655}$ of blank)] × 100. The control represents wells with cells and culture medium, and blank represents wells with culture medium only.

In addition, CCRF-HSB-2 cells were treated with *C. comosum* extract (1.6 μg/ml), and the time course of cell proliferation was measured over the indicated time periods by the WST-1 assay.

Morphological Analysis —— CCRF-HSB-2 cells were incubated with *n*-butanol extract (1.6 μg/ml) in culture medium. After incubation for 1 or 48 hr, the cells were observed under a microscope. Actinomycin D (10 μM) was used as a positive control for apoptosis.

Detection of Apoptosis Induction ——

Analysis of DNA Fragmentation: DNA fragmentation was detected by agarose gel electrophoresis. The extracted residue from *C. comosum* was redissolved in DMSO, and was diluted between 0.4 and 8.0 μg/ml. CCRF-HSB-2 cells (1×10^6 cells) were then incubated with each extract for 2 hr. After the culture medium was replaced, the cells were cultured continuously for 24 hr and then collected. Cellular DNA was extracted by a standard method,[14] and the DNA samples obtained were analyzed by 2% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, and visualized as a DNA ladder with UV. Cisplatin, which induces the apoptosis of CCRF-HSB-2 cells, was used as a positive control.

Detection Using Flow Cytometric Analysis: Apoptosis in cells was detected by terminal deoxynucleotidyltransferase (TdT) and fluorescein isothiocyanate (FITC)-dUTP, referred to as the TdT-mediated nick end-labeling (TUNEL) method,[15] using the MEBSTAIN Apoptosis TUNEL Kit Direct (Medical & Biological Laboratories, Nagoya, Japan). CCRF-HSB-2, HL-60, U937 and HeLa cells (1×10^6 cells) were cultured with the *C. comosum* extract (1.6 μg/ml). After incubation for 72 hr, the cells were washed twice with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then washed with PBS containing 0.2% BSA, and permeabilized with 70% ethanol at –40°C for 2 hr. After being washed, the cells were incubated with FITC-dUTP and TdT for 1 hr. The fluorescence derived from 5000 cells was analyzed by flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

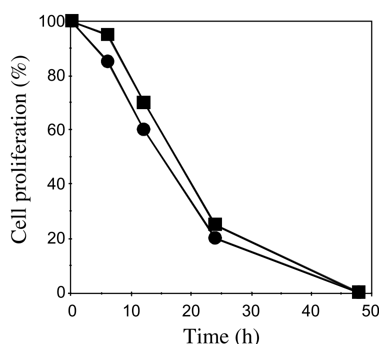

Fig. 1. Cell Proliferation (%) by the Extract from *C. comosum*

CCRF-HSB-2 cells were incubated with extract (1.6 μg/ml) from *C. comosum* (●) or 10 μM Actinomycin D (■). At various times, the CCRF-HSB-2 cells were tested for viability by a WST-1 assay. The values shown at each point are the mean of three independent assays in which three wells each were assessed.

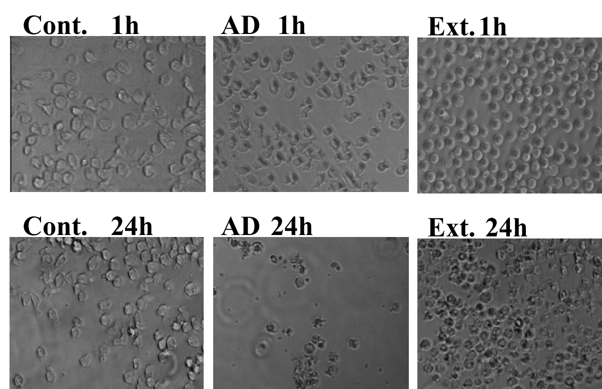

Fig. 2. Morphological Changes in CCRF-HSB-2 Cells Incubated with *C. comosum* Extract (1.6 μg/ml): (Ext.), Control: (Cont.), or 10 μM Actinomycin D: (AD)

Actinomycin D was used as a positive control because it is known to induce apoptosis in various cell lines.[16–18)]

RESULTS

Initially, we examined the cell antiproliferative effects of the extract obtained from the roots of *C. comosum* in human cell lines. The extract showed antiproliferative effects towards HeLa, CCRF-HSB-2, HL-60 and U937 cell lines used in this study, with an IC_{50} value of 6.6, 5.4, 8.7 and 10.2 μg/ml, respectively. The extract showed antiproliferative effects towards all cell linesin a concentration-dependent manner (data not shown). Because the CCRF-HSB-2 cell line was the most sensitive to the *C. comosum* extract in this assay, we used this cell line to test further the precise antiproliferative effects properties of the extract from *C. comosum*.

To investigate its antiproliferative activity further, the extract from *C. comosum* roots was subjected to a cell proliferation assay using CCRF-HSB-2 cells. CCRF-HSB-2 cells were treated with the *C. comosum* extract (1.6 μg/ml), and cell proliferation was examined at intervals by the WST-1 assay. As shown in Fig. 1, the antiproliferation of the cells increased in a time–dependent manner, similar to the antiproliferation of cells treated with actinomycin D.

Morphological changes in the cells caused by the extract from *C. comosum* were observed by microscopy, as shown in Fig. 2. After a 1-hr incubation with the *C. comosum* extract, the CCRF-HSB-2 cells had swelled in comparison to the control cells (Fig. 2, Ext. 1 hr and Cont. 1 hr). After 24 hr, the cells that had died owing to the extract from *C. comosum* were not fragmented, and they maintained a circular form (Fig. 2, Ext. 24 hr). In contrast, there were no morphological changes in the cells incubated with actinomycin D (positive control) for 1 hr (Fig. 2, AD 1 hr). After treatment with actinomycin D for 24 hr, however, the CCRF-HSB-2 cells died and fragmented (Fig. 2, AD. 24 hr).

Next, the CCRF-HSB-2 cells were exposed to a 8.0, 4.0, 0.8 or 0.4 μg/ml *C. comosum* extract for 24 hr and the DNA was extracted. DNA agarose gel electrophoresis was performed and a typical 'DNA ladder' pattern of apoptosis was observed (Fig. 3). This result confirms that extract from *C. comosum* roots can induce apoptosis of CCRF-HSB-2 cells.

Flow cytometric analysis of apoptotic cell death in CCRF-HSB-2 cells, HL-60 cells, U937 cells and HeLa cells using the TUNEL method is shown in Fig. 4. The histograms show the fluorescence staining of DNA breaks in control cells incubated without extract and in cells incubated with *C. comosum* (1.6 μg/ml) extract for 48 hr. It can be seen that all cell lines that were treated with the *C. comosum* extract showed high fluorescence intensity.

DISCUSSION

Mimaki *et al*. have shown that the saponins isolated from *C. comosum* have inhibitory activity by using 12-*O*-tetradecanoylphorbor-13-acetate-stimulated ^{32}P-incorporation into phosholipids of HeLa cells as a primary screening test to identify new an-

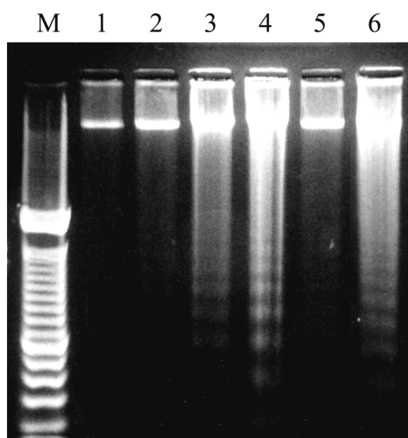

Fig. 3. Analysis of DNA Fragmentation in CCRF-HSB-2 Cells Treated with Extract from *C. comosum* Roots

Electrophoresis of genomic DNA (2×10^5 cells/lane) was carried out on a 2% agarose gel. M, DNA marker; lane 1, normal control; lane 2, 0.4 μg/ml extract for 24 hr; lane 3, 0.8 μg/ml extract for 24 hr; lane 4, 4.0 μg/ml extract for 24 hr; lane 5, 8.0 μg/ml extract for 24 hr; lane 6, 3 μg/ml Cisplatin.

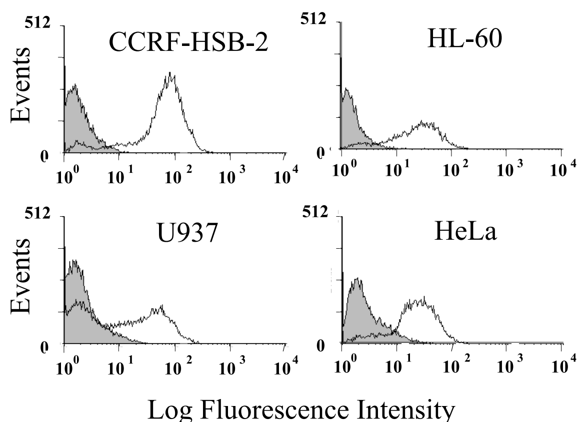

Fig. 4. Detection of Apoptosis Using Flow Cytometric Analysis

Shaded area, control histograms; unshaded area, cells incubated with extract from *C. comosum*.

titumor-promoter compounds.[12] To date, however, there have been no detailed reports about cell death caused by *C. comosum*.

In the present study, we have shown that *n*-butanol extract from the roots of *C. comosum* has antiproliferative activity on CCRF-HSB-2, HeLa, HL-60 and U937 cells in a WST-1 assay. The IC_{50} values of the *C. comosum* extract in CCRF-HSB-2, HeLa, HL-60 and U937 cells were between 5.4 and 10.2 μg/ml, and did not vary much among the cell lines.

To confirm these effects by biochemical analysis, the effects of the extract from *C. comosum* were evaluated further using CCRF-HSB-2 cells. The antiproliferative activity of the extract towards CCRF-HSB-2 cells increases time–dependently, as did the activity of the positive control actinomycin D. Examination of the morphological changes in CCRF-HSB-2 cells treated with the extract from *C. comosum* with a microscope showed that the cells swelled into a circular form after 1 hr and died without fragmentation after 48 hr, which differed from the effects of the positive control actinomycin D (Fig. 2). These results suggest that the *C. comosum* extract contains antiproliferative materials that kill CCRF-HSB-2 cells by a mechanism that differs from that of actinomycin D.

To investigate the relationship between the antiproliferative activity and the apoptosis-inducing activity of the *C. comosum* root extract, DNA was extracted from the treated CCRF-HSB-2 cells and analyzed by DNA agarose gel electrophoresis. A clear, dose–dependent, laddering pattern was observed for the 8.0 and 0.8 μg/ml extract (Fig. 3). The presence of these oligonucleosomal DNA fragments indicates that the CCRF-HSB-2 cells had undergone apoptotic cell death. However, it suggests that there is an optimal concentration to induce apoptosis. Detection of apoptosis using flow cytometric analysis and TUNEL method further indicated that the extract from *C. comosum* also induced apoptosis in HL-60, U937 and HeLa cells (Fig. 4). Taken together, these results show that the antiproliferative effect of the extract is due to the induction of apoptosis.

A number of antitumor agents, such as cisplatin, etoposide, mitomycin, and actinomysin D, have been reported to induce apoptosis in tumor cells. Thus, apoptosis in tumor cells plays a critical role in the killing of tumor cells during cancer chemotherapy.[19]

In summary, we have reported for the first time that the *n*-butanol extract from roots of *C. comosum* induces definite apoptosis in various human cell lines. *C. comosum* has been traditionally used as a folk medicine for cough in China.[11] However, we have found that *C. comosum* contains a new physiological activity that can induce apoptosis in human cell lines. Our results suggest that more research into the induction of apoptosis by components of *C. comosum* is important to assess their potential as new anticancer agents.

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