Effect of Several Species of the Family Rubiacea on Cytotoxicity and Apoptosis in HL-60 cells

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Herbal medicines have been utilized to treat a variety of diseases, including cancer. Several species of the family rubiaceae have been reported to have antitumor activity. In this study, we report the cytotoxicity and antitumor activity exhibited by the methanol extracts prepared from Rubia radix (RRME), Uncaria gambir (UGME) and Oldenlandia diffusa (ODME) (family: Rubiaceae) against human promyleloid leukemia cell line, HL-60. The cytotoxicity of RRME (2 ~ 20 ㎍/㎖), UGME (20 ~ 200 ㎍/㎖) and ODME (20 ~ 200 ㎍/㎖) were assessed by the MTT reduction assay. IC50 values for RRME, UGME and ODME were 11.0, 99.5 and 106.1 ㎍/㎖, respectively. When the HL-60 cells were treated with RRME (10 ㎍/㎖), UGME (120 ㎍/㎖) and ODME (140 ㎍/㎖) for 24 h, several apoptotic characteristics such as DNA fragmentation and morphologic changes were observed. Furthermore, flow cytometric analysis was performed to determine the percent of apoptotic cells. The poupulation of sub-G1 hypodiploid cells was increased 37.49% in RRME treatment, 12.49% in UGME treatment and 7.21% in ODME treatment compared with untreated control cells (2.64%). To further confirm apoptotic cell death, we assayed caspase-3, -8 and -9 activities in RRME, UGME and ODME-treated cells. After treatment of RRME, UGME and ODME for 12 h, caspase-3, -8 and -9 activities significantly increased.compared to untreated control cells. These results show that RRME, UGME and ODME induced apoptotic cell death in HL-60 cells and may have a possibility of potential antitumor activities.

Key words : the family rubiaceae, antitumor activity, apoptotic characteristics, caspase-3, -8 and -9

Introduction

Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms. Vincristine, irinotecan, etoposide and paclitaxel are examples of plant-derived compounds that are being employed in cancer treatment[1]. Numerous plant products in the form of decoction, tincture, tablets and capsules have been clinically used for the treatment of different kinds of cancer[2]. Particularly herbal medicine-based plants have been utilized to treat a variety of diseases, including cancer. Several species of the family rubiaceae have been reported to have antitumor activity[3-7]. Main components of rubiacease plants are alkaloids as terpenoid, anthraquinone and flavonoid[8]. Alkaloids are one of the largest groups of natural products. Several new alkaloids have been isolated from cytotoxic crude extracts. Some of them possess interesting cytotoxic, antitumor or antiparasitic properties.

Herbal medicine on tumor cells have been shown to have a variety of antitumor effects such as cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of matrix metalloproteinases and of tumor invasive behavior. Leukemia is one of the most threatening of the diseases currently in existence[9]. With the recognition that most adult leukemia patients are not candidates for transplantation and a more rational therapy not adequately defined, these individuals are treated with regimens that focus on (or at least include) chemotherapy. Recent studies indicate that a wide variety of chemotherapeutic agents induce apoptotic cell death in susceptible cell lines. Apoptosis is an active mode of cell death, encountered in physiological and pathological cellular situations, and is distinguished from the passive variant, necrosis. Morphological hallmarks of this process include loss of cell volume, hyperactivity of the plasma membrane, and condensation of peripheral heterochromatin, followed by

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cleavage of the nucleus and cytoplasm into multiple membrane-enclosed bodies containing chromatin fragments[10-13]. Human promyleloid leukemia cell lines (HL-60 cells) have proven particularly informative in the study of chemotherapy-associated apoptotic proteolytic events.

In this study, we investigated the cytotoxicity and antitumor activity exhibited by the methanol extracts prepared from Rubia radix, Uncaria gambir and Oldenlandia diffusa (family: Rubiaceae) against human promyleloid leukemia cell line.

Metrials and Methods

1. Chemicals and cell culture

The methanol extracts of Rubia radix (RRME), Uncaria gambir (UGME) and Oldenlandia diffusa (ODME) were obtained from Department of Oncology, Graduate School of East-West Medical Science, Kyunghee University. These methanol extracts were dissolved in DMSO at a concentration 200 ㎎/㎖ and stored at -20℃ and diluted in cell culture medium before use. Caspase substrates (Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA) was obtained from Calbiochem. Human promyleloid leukemia (HL-60) cell lines used in this study was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and routinely cultured under a humidified atmosphere of 5% CO_2-95% air at 37℃in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 200 ㎍/㎖ penicillin, and 100㎍/㎖ streptomycin.

2. Treatment of cells

The methanol extracts (dissolved in DMSO, 200 ㎎/㎖) was used for the treatment of cells. The cells (2×10^5 cells/㎖) were treated with RRME, UGME and ODME at 10, 120 and 140 ㎍/㎖ for 24 h in complete cell medium. The control cells were incubated for 24 h in complete cell medium.

3. Cell viability

Exponentially growing cells were seeded into a 24 well plate at 1×10^5 cells/well in duplicate. The cells were treated with increasing concentrations of the methanol extracts for 24 hr. After the indicated time periods, 100 ㎕ of 5 ㎎/㎖ MTT (Sigma Chemicals Co.) was added to each wells and incubated for 4h. Water-insoluble MTT-formazan crystalswere solubilized by adding equal volume of solubilization solution (10% SDS/0.01 N HCl) and incubating the plate overnight in humidified atmosphere of 5% CO_2 at 37℃. The amount of formazan was determined by ELISA reader (SpectraMAX, Molecular Devices) at 560 ㎚.

4. DNA fragmentation analysis

DNA was purified with the Wizard®Genomic DNA Purification Kit (Promega). Briefly, HL-60 cells (2×10^5 cells/㎖) in 10-Φ dishes were treated with various concentration or time of CDST. Treated cells were washed twice with ice-cold PBS and DNA was purified according to some modified manufacturer's protocol and rehydrated in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) by incubating overnight at 4℃. DNA was analyzed after separation by gel electrophoresis (1.2% agarose gel contained 0.5 ㎍/㎖ ethidium bromide at 100 V for 2 h). DNA bands were visualized under UV transillumiator.

5. DNA content (FACS) analysis

Cell cycle progression and apoptosis were monitored by quantitating cellular DNA content after staing with propidium iodide (PI). Untreated or methanol extracts-treated HL-60 cells were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 100% ethanol for 1 h, washed with PBS, and then treated with 10 ㎍/㎖ PI (Sigma) /100 ㎍/㎖RNase A (Sigma) solution at 37℃ for 1 h in the dark, and analyzed on a fluorescence-activated cell sorter flow cytometer (FACS Calibur, BD Biosciences). The data were registered on a logarithmic scale. Apoptotic cells were quantified on the PI histogram as a hypodiploid peak. Cell cycle analysis was determined DNA content from fixed cells stained with PI.

6. DAPI staining

The HL-60 cells (2×10^5 cells/㎖) were cultured in 6-well plate in RPMI 1640 medium and HL-60 cells were incubated with methanol extracts for 24 h. Cells were attached to slides by cytospin, washed with DAPI-methanol (1 ㎍/㎖, Roche), and then stained with DAPI-methanol for 15 min at 37℃. After, stained cells were washed once with methanol and observed under a fluorescence microscope.Apoptotic cells were identified by features characteristic of apoptosis (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies)

7. Caspase activity assay

Cells were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1mM DTT, 0.1 mM EDTA) and lysed by freezing/thawing. Cells lysates were obtained after centrifugation (10,000 rpm) for 1 min at 4℃. Caspase activity assay was performed following the manuufacturer's instructions. In brief, the whole reaction contained 10 ㎕ cell lysates (30 ㎍ total protein), 88 ㎕ reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS,

10mM DTT, 0.1 mM EDTA, 10% glycerol), and 2 μℓ fluorogenic Ac-DEVD-pNA, Ac-IETD-pNA or AC-LEHD-pNA (Calbiochem) substrate (200 μM final concentration). Samples were incubated for 3 h at 37℃ and enzyme-catalyzed release of p-nitroanilide was monitored at 405 ㎚ in ELISA reader.

Results

1. Cytotoxic effect of RRME, ODME and UGME in HL-60 cells

The effect of RRME, ODME and UGME on the cytotoxicity of cells was evaluated using the MTT assay. Shown as Fig. 1, a 24 h exposure to these methanol extracts dramatically decreased the viability of HL-60 cells in a dose-dependent manner. The concentration required to inhibit growth of HL-60 cells by 50% (IC_{50}) was approximately 11.0 μg/㎖ in RRME, 99.5 μg/㎖ in ODME and 106.1 μg/㎖ in UGME. These results suggest that methanol extracts of rubiaceae have cytotoxicity about HL-60 cells.

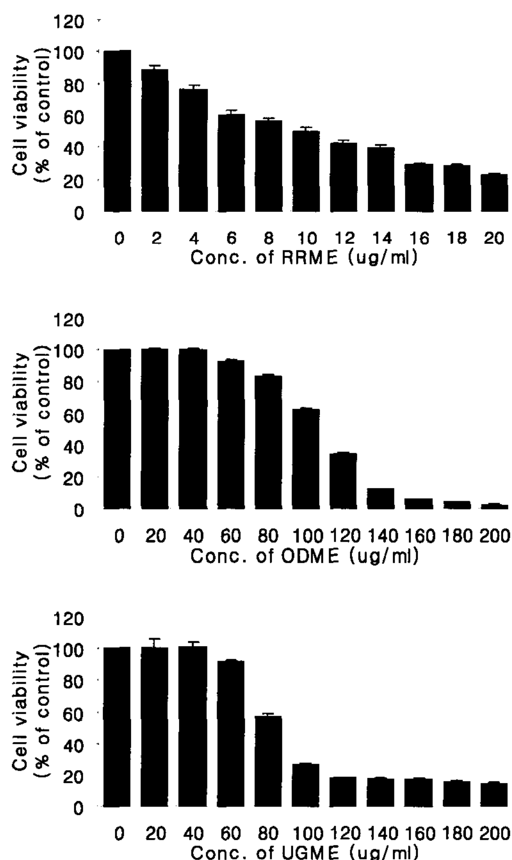

Fig. 1. The effect of RRME, UGME and ODME on cytotoxicity in HL-60 cells. The cells were treated with various concentrations of methanol extracts for 24 h and the cells were tested for viability by MTT assay. Value are means ± SD, N = 3

2. Morphological changes in HL-60 cells treated with RRME, ODME and UGME

The morphology of cells treated with methanol extracts of rubiaceae was studied to test whether cell death was a result of apoptosis in HL-60 cells. Morphological characteristics of apoptosis, including nuclear condensation and fragmentation, were observed under fluorescent microscope by DAPI staining. The bright blue apoptotic nuclei were readily identified by their condensed chromatin and apoptotic bodies. HL-60 cells were treated with RRME (10 μg/㎖), ODME (120 μg/㎖) and UGME (140 μg/㎖) for 24 h. These methanol extracts increased in the number of apoptotic cells in HL-60 cells (Fig. 2). Taken together, along with the appearance of elongated cells, disintegrated cells, as evidenced by apoptotic bodies, and cells with condensed nuclear chromatin appeared in response to methanol extracts of rubiaceae treatment.

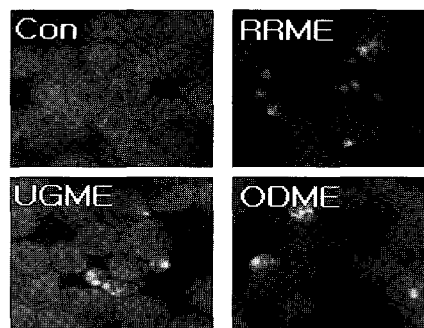

Fig. 2. The effect of N. Rhizoma on morphology in HL-60 cells. The cells were treated with RRME (10 ㎍/㎖), UGME (120 ㎍/㎖), ODME (140 ㎍/㎖) for 12 h. After, cells were subjected to cytospin, stained with DAPI-MeOH and observed under a fluorescence microscope.

3. RRME, ODME and UGME induces apoptotic cell death in HL-60 cells

The biochemical hallmark of apoptosis in degradation of DNA by endogenous DNase, which cut the internucleosomal regions into double-stranded DNA fragments of 180~200 base pairs. We examined the effects of RRME, ODME and UGME on the internucleosomal DNA fragmentation in HL-60 cells through agarose gel electrophoresis. As shown in Fig. 3, these methanol extracts induced DNA fragmentation in HL-60 cells. To further determine the degree of apoptosis of HL-60 cells treated with methanol extracts for 24 h, we employed flow cytometry to quantify the sub-G1 peak (apoptotic peak). Fig. 4 shows that the rate of apoptotic cells increased to 37.49, 12.49 and 7.21% after treatment with RRME, ODME and UGME, respectively. Flow cytometric analysis of methanol extracts of rubiaceae treated HL-60 cells showed the increase of hypodiploid apoptotic cells. These results suggest that methanol extracts of rubiaceae can trigger apoptosis of HL-60 cells.

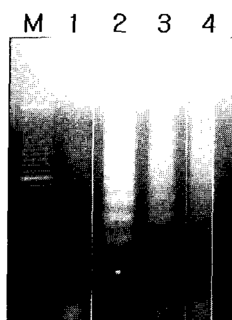

Fig. 3. The effect of N. Rhizoma on DNA fragmentation in HL-60 cells. The cells were treated with RRME (10 ㎍/㎖), UGME (120 ㎍/㎖) and ODME (140 ㎍/㎖) for 24 h. DNA was extracted, then separated by agarose gel (contained EtBr) electrophoresis, and visualized under UV light. M, 100 bp DNA ladder marker; Lane 1, untreated control; Lane 2, RRME treatment; Lane 3, UGME treatment, Lane 4, ODME treatment.

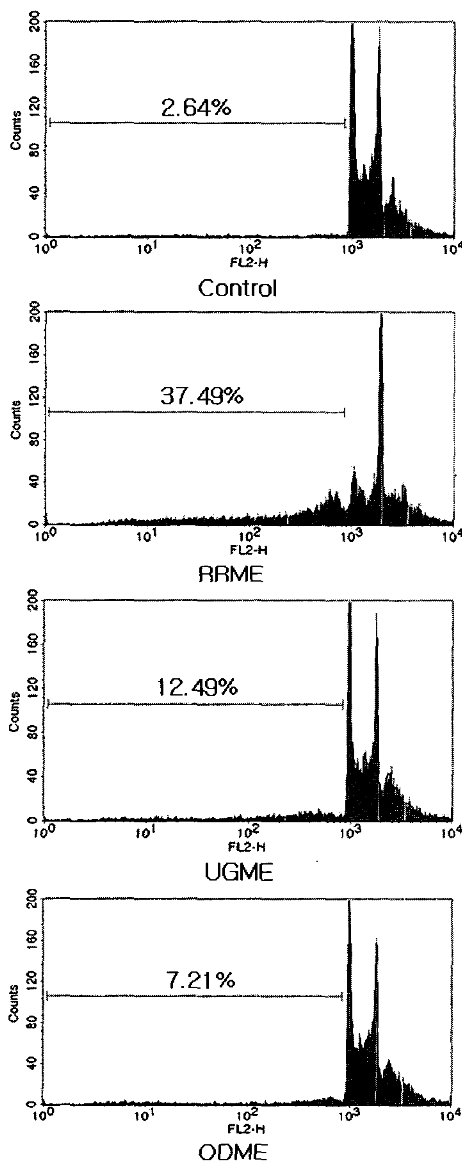

Fig. 4. The effect of RRME, UGME and ODME on the DNA content in HL-60 cells. The cells were exposed to RRME (10 ㎍/㎖), UGME (120 ㎍/㎖) and ODME (140 ㎍/㎖) for 24 h, wahsed and then harvested. The cells were fixed and stained with PI and the DNA content was analyzed by FACS.

4. RRME, ODME and UGME induces activation of caspase-3, -8 and -9

Caspase family plays key roles in the execution of apoptotic cell death. Of these, caspase-3, -8 and -9 is the most important cell executioners for apoptosis. We observed the proteolytic activation of procaspase-3, -8 and -9 induced by the RRME, ODME and UGME. To determine whether activation of caspase-3, -8, and -9 plays a role in methanol extracts of rubiaceae-induced apoptosis, HL-60 cells were incubated with methanol extracts for 12 h, and the cleavage of the p-nitroanilide from the specific fluorogenic substrate for caspase-3 (Ac-DEVE-pNA), -8 (Ac-IETD-pNA) and -9 (Ac-LEHD-pNA) were analyzed colorimetrically. The activity of caspase-3, -8 and -9 were all significantly increased in compared with untreated-control (Fig. 5). These results show that methanol extracts of rubiaceae induced apoptotic cell death in HL-60 cells through activation of caspase-3, -8 and -9.

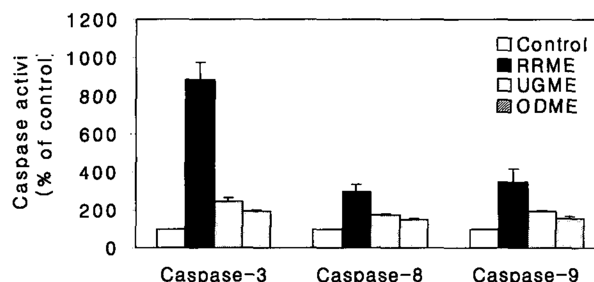

Fig. 5. The effect of N. Rhizoma on activation of caspase-3, -8 and -9 in HL-60 cells. HL-60 cells were treated with RRME (10 ㎍/㎖), UGME (120 ㎍/㎖), ODME (140 ㎍/㎖) for 12 h. Caspase-3, -8, and -9 activities were measured with AC-DEVD-pNA, AC-IETD-pNA, and AC-LEHD-pNA respectively. The activation was measured as the increase in activity (%) with respect to control cells (100%). Value are means ± SD, N = 3.

Discussion

In this study, we have examined the cytotoxic effect of RRME, ODME and UGME, methanol extracts of rubiaceae, in HL-60 cells. We have shown that thease methanol extracts was cytotoxic to HL-60 cells; the IC50 value was approximately 11.0 ㎍/㎖ at RRME, 99.5 ㎍/㎖ at ODME and 106.1 ㎍/㎖ at UGME (Fig. 1). Analysis of cell morphology, DNA fragmentation, and caspase activity in HL-60 cells incubated with methanol extracts suggest that the cytotoxicity of methanol extracts was mediated by the induction of apoptosis. Apoptosis is an important phenomenon in cancer chemotherapy, because anticancer drugs exert their antitumor effect against cancer cells by inducing apoptosis. The general feature of apoptosis is apoptotic body and DNA fragmentation. Typically, the apoptotic body was characterized by nuclear

condensation and cell shrinkage, and DNA fragmentation was formed by cleavage of DNA of apoptotic cells, which is population of multimers of 180~200 bp fragments. After HL-60 cells were treated with methanol extracts for 12 or 24 h, we have observed apoptotic bodies in morphological analysis of apoptotic cells with DAPI staining (Fig. 2) and confirmed DNA fragment ladder formation through gel electrophoresis (Fig. 3). Apoptosis is mainly brought about by activation of caspases, a protease family with unique substrate selectivity. It is known that caspase-3, which is the main executioner caspase, can be activated by caspase-8 and/or caspase-9[14]. It depends upon the apoptosis-inducing stimulus whether caspase-3 is activiated by both/either caspase. As shown in Fig. 5, methanol extracts of rubiaceae have increased the caspase-3 by both caspase-8 and caspase-9, but how these methanol extracts activates caspase-3 is not clear. Because activation of caspase-3 mediated by two pathways consisting of the initiator caspases, caspase-8 or caspase-9. Apoptosis is cellular suicide or programmed cell death that is mediated by the activation of an evolutionary conserved intracellular pathway. The signaling pathway of apoptotic cell death is diverse. For the apoptotic process, 3 major pathway have been established; signal caused by apoptotic stimulus acts on mitochondria to release cytochrome c to activate caspase (intrinsic pathway)[15], mitochondrion-bypass pathway that activates caspase (extrinsic pathway)[16], caspase-independent pathway[17]. Apoptotic pathway of HL-60 cells by methanol extracts of rubiaceae is not yet clear. We are need of further studies that examine the signaling pathway of apoptotic cell death by RRME, ODME and UGME.

Recent approach in the therapy of leukemia includes the use of differentiation inducing agents such as interferon[18], retinoids[19], and 1α,25-dihydroxyvitamin D3[20,21]. Based on this, the strategy of HL-60 cell differentiation has been accepted as a valid model in detecting or screening for potential cancer chemopreventive and/or chemotherapeutic agents in preclinical evaluation. We examined the effects of RRME, ODME and UGME on differentiation of HL-60 cells, but these methnol extracts was no effect on differentiation of HL-60 cells (data not shown).

In conclusion, RRME, ODME and UGME were shown the cytotoxicity and apoptosis in HL-60 cells. But, these methanol extracts mechanism of action remains unknown.

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