Apoptotic Effect of *Rubia cordifolia* Dichloromethane Extracts on Human Acute Jurkat T Cells

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To understand cytotoxic activity of *Rubia cordifolia L.* (Rubiaceae), which has been used as a traditional oriental medicine, the mechanism underlying cytotoxic effect of its extract on human acute Jurkat T cells was investigated. The methanol extract of roots (3 kg) of *R. codifolia* was evaporated, dissolved in water, and then extracted by dichloromethane. The substances in the chloroform extract showing the most cytotoxic activity were further purified by a series of preparative HPLC. The extracted active substance (65 mg) was designated as CCH1. When Jurkat T cells were treated with CCH1 at concentration ranging from 0.5 to 2.0 μg/ml, apoptotic phenomena of cells companying several subsequent biochemical reactions such as mitochondria cytochrome c release, activation of casapase-8, -9, and caspase-3, degradation of PARP and DNA fragmentation occurred via mitochondria-dependent pathway. However, abrogation of apoptosis was observed in an ectopic expression of Bcl-xL, which is a suppressor for mitochondrial cytochrome c release. These results demonstrate that the cytotoxicity of CCH1 against Jurkat T cells is attributable to apoptosis mediated by mitochodria-dependent death-signaling regulated by Bcl-xL. In addition, the CCH1 is more potent to leukemia Jurkat T cell than to human peripheral blood monocyte cells (PBMC).

Key words : Anticancer, apoptosis, cytotoxicity, Jurkat T cells, *Rubia cordifolia*

Introduction

Rubia cordifolia L. (Rubiaceae) was one of the best candidates. *R. cordifolia* have been reported for an important oriental medicine plant which is used for anti-inflammatory [1], immunomodulatory [6], anticonvulsant and anxiolytic [8] and anti-tumor activity [13]. It has reported that cyclic hexapeptide isolated from *R. cordifolia* (Rubiaceae) shows high anti-tumor activity [4]. However, anti-tumor effect of other chemicals isolated from *R. cordifolia* has not been elucidated. We have found that a potential cytotoxic substance except hexapeptide also exists in roots of *R. cordifolia.* The substance was partial purified, and designated as CCH1. In this report, we investigated signaling pathways during CCH1-induced apoptosis in Jurkat T cells, with particular focus on mitochondria-dependent cell death and whether it could be protected by the ectopic expression of anti-apoptotic protein, Bcl-xL. These results showed that the CCH1-induced apoptotic cell death was mediated by mitochondrial cytochrome c release with resultant activation of caspase-8, -9 and -3, and cleavage of poly (ADP-ribose) polymerase (PARP), which was suppressed by Bcl-xL.

In the present study, we investigated approximately 80 medicine plants to find potent cytotoxicity material toward human Jurkat T cells.

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Material and Methods

Isolation of a cytotoxic component from *Rubia cordifolia*

*Rubia cordifolia Lin*n. (Rubiaceae) was purchased from Hyun-dae Pharmacological Company (Busan, Korea). A sample has been deposited in the author's laboratory (Kim). The dried roots (3 kg) from *R. cordifolia* were extracted with 100% methanol. The methanol extract was evaporated (295.2 g), dissolved in water, and then extracted with dichloromethane (CH_2Cl_2). The CH_2Cl_2 fraction (82.5 g) had cytotoxicity against Jurkat T cells. The fraction was then subjected to silica gel column chromatography using a solvent system of CH_2Cl_2:EtOAc (50:1) to afford 60 fractions (100 ml/fraction). Analysis of each fraction was performed by a thin layer chromatography (TLC, 20 cm×20 cm, kiesel 60 F254, Merck, Germany) with a solvent system (CH_2Cl_2:EtOAc=

50:1), and the separated spots on the TLC were visualized with UV irradiation and 50% H_2SO_4. The fractions having the same pattern of separated spots on the TLC were collected together, and the cytotoxic activity of each fraction against Jurkat T cells was determined by MTT (Sigma, USA) assay. The active fraction (4.1 g) was applied on secondary silica gel column chromatography using a solvent system (n-hexane:ethyl acetate=10:1), and subsequently on a preparative HPLC GS320 column (Japanese Engineering Instrument Co., Japan) chromatography (eluted with CH_3CN: H_2O=10:1, 3.0 ml/min, 245 nm). The active fraction by the HPLC GS320 column chromatography afforded 65 mg of cytotoxic compound, of which the IC50 value was 1.7 μg/ml against Jurkat T cells. The cytotoxic compound was designated as CCH1. The IC50 was expressed as concentration of sample that inhibits 50% of growth compared with a drug-free control at 37°C for 20 hr.

Reagents, antibodies, and cells

ECL Western-blotting kit was purchased from Amersham Biosciences (Arlington Heights, IL, USA). Antibodies used in this study were anti-cytochrome c (Pharmingen), anti-caspase-8, anti-caspase-9 (Cell signaling Technology, N.K, USA), anti-caspase-3, anti-PARP and anti-β-actin (Santa Cruz Biotechnology, Inc., USA). A broad-range caspase inhibitor z-VAD-fnk was obtained from Calbiochem (San Diego, CA, USA). The human T cell lymphoma line, Jurkat T E6.1 cells were obtained from Albert A Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA). The Jurkat T E6.1 cells were grown in RPMI1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), β-mercaptoethanol, and 100 μg/ml G418 (Invitrogen, USA). Expression vectors for J/Neo and J/Bcl-xL were a gift from Dennis Taub (Gerontology Research Center, NIA/NIH, MD, USA).

Cytotoxicity assay with Jurkat E6.1 T cell

Cytotoxicity of CCH1 against Jurkat T cell was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay as described previously [2]. Briefly, the cells (4×10^4) were added to a serial dilution of the CCH1 in 96-well plates. After incubation for 20 hr, 50 μl of the MTT solution (1.0 mg/ml) was added to each well and incubated for an additional 4 hr. After centrifugation, the supernatant was removed from each well and then 150 μl of demethyl sulfoxide (DMSO) was added to dissolve the colored formazan crystal produced from the MTT. The OD values of the solutions were measured at 540 nm using a plate reader. Viable cells were counted by a haemocytometer with trypan blue staining [7]. For the trypan blue assay, cells were cultured in a 35 mm dish and exposed to various concentration of CCH1 for 20 hr. The cells were washed with phosphate buffered saline (PBS), and trypan blue dye solution was then added to the cell suspension. After that, the viable cells were counted with a haemocytometer.

Immunoblotting

Cells pretreated with or without CCH1 were lysed with lysis buffer [137 mM NaCl, 15 mM EDTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25mM 3-N-morpholino-propanesulfonic acid (MOPS, Sigma, USA), and 2.5 μg/ml proteinase inhibitor E-64, pH 7.2]. Cell suspension was sonicated for 30 min at 4°C and centrifuged. Protein concentration was measured by BCA (Peirce, USA). After 4-12% gradient SDS-PAGE, the samples were transferred onto immobilon-P membranes. The membranes were soaked in a blocking solution (5% skim milk and 0.2% Tween 20-PBS) for 1 hr, and then incubated with primary antibodies. After being washed with Tween 20-PBS, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 hr. Specific bands were visualized by an ECL method (ECL+Amersham Biosciences)

Detection of cytochrome c released from mitochondrial cytosol

To detect cytochrome c, cytosolic proteins of CCH1 treated cells were extracted as described elsewhere [15]. Briefly, cells were harvested, washed with ice-cold PBS, and then incubated with 500 μl of lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2.5 μg/ml proteinase inhibitor E-64, 20 mM HEPES, pH 7.2) on ice for 30 min. Cells were passed through the 26-G needle with 20 strokes. Then, the disrupted cells were centrifuged at 750 g for 10 min. The supernatant was centrifuged at 10,000 g for 25 min. After centrifugation, cytosolic fraction was frozen at -70°C. Total lysates were used as the sample of the cytochrome c release.

Results

CCH1 has the cytotoxicity against Jurkat T cells

We tested CCH1 for cytotoxicity by using Jurkat T cells.

Fig. 1. Effect of CCH1 on viability and apoptotic phenomea in Jurkat T cells. CCH1 shows cytotoxicity dose-dependentlly in Jurkat T cells. Continuously growing Jurkat T cells (4×10^4) were incubated with indicated concentrations of CCH1 in a 96-well plate for 24 hr and further incubated with MTT for 4 hr. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability.

Fig. 2. CCH1 facilitates the activation of mitochondria-dependent death-signaling in Jurkat T cells. (A) Apoptosis of Bcl-xL over-expressed cells have no influence on CCH1. J/Bcl-xL and J/Neo cells (4×10^4 cells) were incubated with indicated concentrations of CCH1 in microplate for 20 hr. The cells were further incubated with DMSO for 4 hr to solve the colored formazan crystal produced from MTT as an index of cell viability.

The cells (4×10^4) were incubated with CCH1 solution containing 0.5-2.0 μg/ml concentration in 96-well plate for 24hr. The CCH1 (0.5 μg/ml) treated-cells showed the cell viability about 90% and the CCH1 (2.0 μg/ml) treated-cells showed the cell viability less than 50% (Fig. 1). This means that CCH1 induces the apoptosis of Jurkat T cells.

Apoptosis is abrogated in J/Bcl-xL cells treated with CCH1.

Bcl-xL is well known to block a cell apoptosis phenomenon [10]. We tested protection of cell apoptosis in Bcl-xL over-expressed cell (J/Bcl-xL) stimulated with CCH1. Cells (4×10^4) treated at concentration ranging 0.5 to 2.0 μg/ml CCH1 were incubated for 20 hr and carried out with MTT assay. As shown in fig. 2, 0.5 μg/ml treated-control cells (J/Neo) show cell viability about 90%, but cell viability was decreased dose-dependently with increased CCH1 concentration. However, J/Bcl-xL cells show no significant cell viability changes at indicated several concentrations (Fig. 2). These results show that over-expressed Bcl-xL proteins abrogate cytochrome c release from mitochondria and subsequent activation of apoptosis signaling. Therefore, these experiments demonstrate that CCH1 facilitates the activation of mitochondria-dependent death-signaling pathway in Jurkat T cells.

Apoptosis pathway triggered by CCH1 is through mitochondrial cytochrome c release

We investigated the apoptosis mechanism by utilizing dose-dependent treated J/Bcl-xL cells and J/Neo cells stimulated by CCH1 to find molecular mediators. Significant differences from antibody reaction related to mitochondrial cytochrome c release pathway [14] were detected in J/Neo cell compared to J/Bcl-xL cells. Release level of mitochondrial cytochrome c to cytosol was increased in J/Neo cells incubated with 0.5-2.0 μg/ml of CCH1 (Fig. 3A). Subsequently, released cytochrome c switched pro-caspase 9, downstream partner of cytochrome c related to apoptotic pathway, to active-caspase 9, dose-dependently (Fig. 3B) in J/Neo cells. The amount of active-caspase 3 was increased in the range from 1.0 μg/ml to 2.0 μg/ml of CCH1 (Fig. 3D) in J/Neo cells. Next, we performed Western blotting for PARP, which is the downstream target of active-caspase 3. As pro-caspase 3 ranged from 1.0 μg/ml to 2.0 μg/ml of CCH1 was activated, PARP was degraded (Fig. 3E) in J/Neo cells. In this study, it was found that cleavages of caspase-8 and -9 in Jurkat T cells were also induced by CCH1 treatment (Fig. 3B, 3C). Recent reports [9,17] suggest that pro-caspase-3 can be activated through active caspase-8 in turn can cleave Bid, leading to cytochrome c release from mitochondria [12] and setting up a self-amplification loop to amplify caspase-9. Together, these findings support the notion that apoptotic signaling of CCH1 in Jurkat T cell is regulated by mitochondrial cytochrome c release pathway.

Cytotoxic effect of CCH1 on PBMC

Cytotoxic effect of CCH1 was tested with normal PBMC

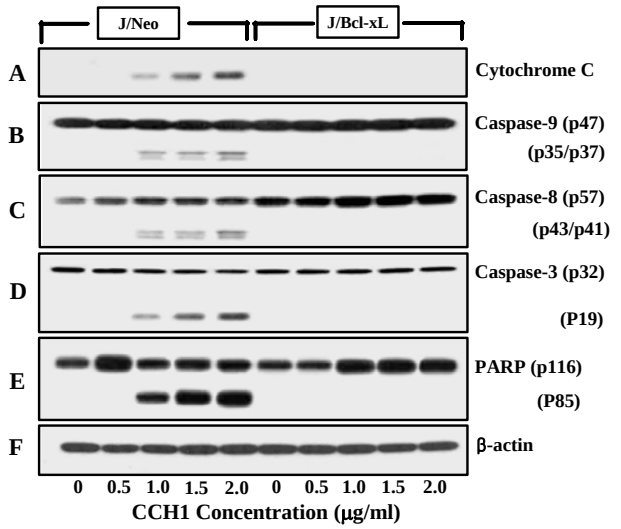

Fig. 3. CCH1 induced-cell death is through mitochondrial cytochrome c release. The cells (5×10^6) were incubated with indicated concentrations of CCH1 for 20 hr and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4~12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in materials and methods using the PIERCE Western blot detection system. Cytochrome c (A), caspase-9 (B), caspase-8 activation (C), caspase-3 activation (D), cleavage of PARP (E), and β-actin (F).

at concentration ranging from 0 to 130 μg/ml for 48 hr. As shown in fig. 4, cell viability of Jurkat T cells was inhibited by 70% at 8.0 μg/ml of CCH1, however, PBMCs were dead by 34% at the same concentration of CCH1.

Thus, acute Jurkat T lymphoma showed sensitivity to CCH1, but normal PBMCs were resistant against CCH1. It seems that CCH1 is used as evaluation for chemotherapeutic agent, or tonic material for human health.

Fig. 4. Comparative cell viability of PBMCs and Jurkat T cells treated with CCH1. PBMCs and Jurkat T cells were introduced at a density of 2.5×10^5 cells in each well of 96-well plate and cultured for 48 hr. Cell viability was calculated by MTT assay.

Discussion

In this research, we have first demonstrated that a partial purified phytochemical ingredient designated as CCH1 from *R. cordifolia*, which has been used as tonic materials or fork remedies in several countries including Korea, stimulates human acute Jurkat T cell apoptosis via mitochondria dependent caspase signaling. With dried roots (3 kg) of *R. cordifolia*, the purification procedure employed in this study finally afforded 65mg of CCH1 as described in material and methods. Further analysis to examine the purity of CCH1 by analytical HPLC C18 column chromatography revealed that CCH1 is composed of the mixture of two substances (data not shown). The CCH1 appear to be a steroid substance because it positively reacts with antimony trichloride-$CHCl_3$ based on spraying chemical reagent on TLC, but it fails to react with ninhydrine and $FeCl_3$. In addition, CCH1 appeared to contain carbohydrate because of positively reacting with anisaldehyde, 2,4-dinitrophenyl hydrazine, and aniline phthalate reagents on TLC. Taken together with these chemical properties, it seems that CCH1 is a steroid derivative. For the chemical structure and pure substance, further instrument analysis is on track. Cytotoxicity of *R. cordifolia* against acute Jurkat T cell lymphoma was expressed as IC_{50} during purification, and the IC_{50} was determined at each step fractionated. The results showed that IC_{50} at methanol extract, CH_2Cl_2 fraction, silica gel chromatography, and preparative HPLC GS320 chromatography was 65 μg/ml, 32 μg/ml, 6.3 μg/ml, and 1.7 μg/ml, respectively. Although it has been reported that bicyclic hexapeptides (RA-IX and RA-Z) in *R.cordifolia* have potential anticancer activity [4], but little is known that other biochemical including a steroid derivative in *R. cordifolia* shows potential cytotoxicity against cancer cells, and especially signaling pathways during apoptosis is still unknown. In this report, we have understood the cytotoxic effect, the apoptotic signaling pathway, and cell cycle distribution of CCH1 by taking advantage of antiapoptotic protein, Bcl-xL whose major function is known to suppress mitochondrial cytochrome *c* release [3,14]. In Jurkat T cells exposed to CCH1, mitochondrial cytochrome c release, activation of caspase-3, -8 and -9, and cleavage of PARP were detected. These results indicated that CCH1-induced apoptotic DNA fragmentation was triggered by mitochondria-dependent death-signaling

pathway including cytochrome c release and caspase activation. Recent reports [9,16] suggest that caspase-8 activation, when triggered downstream of the mitochondrial pathway of apoptosis, may amplify caspase-9 activation through the cleavage of the pro-apoptotic protein Bid, which, in turn, elicits a further efflux of cytochrome c from mitochondria [12]. Although the current results indicate that CCH1-induced apoptosis is accompanied by mitochondrial cytochrome c release and subsequent activation of caspase cascade in Jurkat T cells, we employed two experimental approaches to confirm that these cellular events are prerequisite for the CCH1-induced apoptosis; one is for taking advantage of antiapoptotic protein Bcl-xL whose major function is known to suppress mitochondrial cytochrom c release [11,18] and the other is to use z-VAD-fmk, a well known broad-range caspase inhibitor [17]. The CCH1-induced apoptosis was abrogated by an ectopic over-expression of Bcl-xL. In addition, sensitivity of Jurkat T cells on CCH1 is more than that of normal PBMCs.

Collectively, these results demonstrate that CCH1, a partially purified constituent from medicinal plant *R. cordifolia*, induces apoptosis of Jurkat T cells via mitochondria dependent pathway by release of mitochondria cytochrome *c*, activation caspase-8, -9, and subsequent cascade events. These things will be helpful and useful for evaluation of its chemotherapeutic potency and tonic material.

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초록 : 천초근 dichloromethane 추출물의 Jurkat T 세포에서 세포사멸 효과

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천초근은 전통적으로 동양의학에서 항암제로 사용되어왔는데 인간 급성 백혈병 세포주인 Jurkat T 세포를 사용하여 천초근의 세포독성기작을 알아보았다. 천초근 뿌리(3 kg)를 메탄올로 추출, 증류한 후 물에 녹여 다시 dichloromethane으로 추출분획 하였다. 세포독성 활성을 보이는 dichloromethane 추출물을 연속적으로 HPLC를 통해 분리하였고 그 활성물질(65 mg)을 CCH1이라 명명하였다. CCH1을 0.5 μg/ml에서 2.0 μg/ml의 농도로 처리하고 세포사멸 과정을 보았다. 즉, mitochondria cytochrome c 방출, casapase-8, -9 및 caspase-3의 활성화, PARP 분해, DNA 단편화 현상들이 일어나는 것을 관찰하였다. 하지만, mitochondria cytochrome c 방출 억제자인 Bcl-xL이 과발현되는 Jurkat T 세포에서는 세포사멸현상이 일어나지 않았다. 이러한 결과는 CCH1이 mitochondria 의존적인 신호전달 과정을 통해서 세포사멸을 유도 한다고 할 수 있다. 그리고 CCH1에 의한 세포독성은 혈액에서 분리한 단핵구 세포보다 Jurkat T 세포에서 보다 강한 활성을 보였다.