Study on the Antileukemic Effect of Galla Rhois

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Galla Rhois is a nest of parasitic bug, *Mellaphis chinensis Bell*, in *Rhus chinensis Mill*. Galla Rhois has been used for the therapy of diarrhea, peptic ulcer, hemauria, etc., that showed various antiinflammatory activity, and other biological properties. We studied the effect of Galla Rhois water extract(GRWE). The cytotoxic activity of GRWE in HL-60 cells was increased in a concentration-dependent manner. GRWE was cytotoxic to HL-60 cells, with IC_{50} of 100 ㎍/㎖. Treatment of GRWE to HL-60 cells showed the fragmentation of DNA in a concentration manner, suggesting that these cells underwent apoptosis. In addition, the flow cytometric analysis revealed GRWE concentration-dependently increased apoptotic cells with hypodiploid DNA content and arrested G1 phase of cell cycle. These results indicate that GRWE may have a possibility of potential anticancer activities. Treatment of HL-60 cells with GRWE was induced activation of caspase-3, caspase-8 and proteolytic cleavage of poly(ADP-ribose) polymerase. Also, caspase-3 was directly activated via caspase-8 activation. GRWE also caused the release of cytochrome c from mitochondria into the cytosol. GRWE-induced cytochrome c release was mediated by caspase-8-dependent cleavage of Bid and Bax translocation. These results suggest that caspase-8 mediates caspase-3 activation and cytochrome c release during GRWE-induced apoptosis in HL-60 cells.

Key words : Galla Rhois water extract(GRWE)

Introduction

Many medicinal herbs showed the antitumoral activity[1]. Some anti-cancer agents cause cell deaty by apoptosis.[2-6] Apoptosis is known as programmed cell death and follows the activation of signal transduction, and a selective physiological process that plays an important role in the blance between cell proliferation and cell death.[7-9] Apoptosis is an important phenomenon in cancer cheomtherapy, because anti-cancer agent exert their antitumor effect against cancer cells by inducing apoptosis.[10]

The signaling pathway of apoptotic cell death is also diverse, in other words, the pathway used is greatly affected by the kind of stimuli. For the apoptotic process, 3 major pathways have been established: signal caused by apoptotic stimulus act on mitochondria to release cytochrome c to activate caspase (chemical-induced apoptosis),[11] mitochondrion-bypass pathway that activates caspases (receptor-induced apoptosis),[12] caspase-independent pathway.[13]

Apoptotic stimuli such as activation of cell surface receptors or environmental stress can induce cytochrome c release from mitochondria. Once released, cytochrome c binds to Apaf-1 and activates caspase-9 in the presence of dATP.[14-16] The activated caspase-9 leads to the activation of downstream effector caspase, such as caspase-3, which cleaves a number of cellular proteins to execute cell death. it has recently been proposed that in receptor-mediated apoptosis, Bid, activated by caspase-8, is translocated to the mitochondria and induces the release of cytochrome c, whereas in chemical-induced apoptosis, cytochrome c release is caspase-independent and is not mediated by cleavage of Bid.[17-19].

Bax is proapoptotic members of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis.[20-22] Recently, Bax has been shown to induce cytochrome c release and caspase activation in vivo and in vitro.[21,23]

Mechanisms for the release of mitochondrial cytochrome c include opening of a mitochondrial permeability transition pore, the presence of a specific channel for cytochrome c in the outer membrane, or mitochondrial swelling and rupture of the outer membrane but without loss of mitochondrial membrane

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potential.[24] None of these mechanisms appears generally applicable, as release of cytochrome c occurs in cells with normal mitochondrial membrane potential[25,26] and by a mechanism independent of rupture of the outer mitochondrial membrane.[27] Two recent studies have highlighted another possible mechanism of mitochondrial cytochrome c release, involving Bid, a BH3 domain-containing propoptotic Bcl-2 family member. Cleavage of Bid by caspase-8 results in translocation of the celaved Bid to the mitochondria where it induces the release of cytochrome c, being 500-fold more potent than Bax.[28,29] The BH3 domain of Bid is essential both for its proapoptotic activity and its ability to induce the release of cytochrome c.[29,30]

In this study, we investigated the cellular mechanisms of cell death in HL-60 cells induced by GRWE. We showed that GRWE induced apoptotic cell death in HL-60 cells by caspase activation following cytochrome c release. It demonstrated that GRWE -induced apoptotic cell death activated caspase-3 through caspase-8, and released cytochrome c from mitochondria via Bid cleavage and Bax translocation in HL-60 cells.

Materials and Methods

1. Cell culture

The lymphoma (U-937) and leukemia (Jurkat, Molt-4, HL-60) cell lines used in this study was obtained from Korean Cell Line Bank (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. Preparation of Galla Rhois water extract (GRWE)

The plant sample was obtained from Oriental Medical Hospital, Wonkwang University. Botanical identification was confirmed at the Herbarium of the College of Oriental Medicine, and the specimen was deposited with voucher number OM-PH-009. An aqueous extract of Galla Rhois was prepared by decocting the dried prescription of herbs with boiling distilled water for 3 h. The decoction was filtered, lyophilized, and kept at 70C. The yield of dried extract from starting crude materials was about 10%.

3. Cell viability

Exponentially growing cells were seeded into a 24 well plate at 2×10^5 cells/㎖ in duplicate. The cells were treated with increasing concentrations of GRWE for 24 hr. After the indicated time periods, 10 ㎎/㎖ MTT was added to each wells and incubated for 4 hr. Water-insoluble MTT-formazan crystals were solubilized by adding solubilization solution (10% SDS/0.01N HCl) and incubating the plate overnight in humidified atmosphere of 5% CO_2 at 37℃. The amount of formazan was determined by measuring the absorbance an 570 ㎚.

4. DAPI staining

The HL-60 cells (2×10^5 cells/㎖) were cultured in 6-well plate in RPMI 1640 medium and treated with increasing concentration of GRWE for 24 h. Cell were then stained with DAPI-Methanol (1㎍/㎖, Roche). After, stained cells were attached to slides by cytospin and observed under a fluorescence microscope.

5. DNA fragmentation analysis

DNA was purified with the Wizard® Genomic DNA Purification Kit (Promega). Briefly, HL-60 (2×10^5 cells/㎖) cells in 10-Φ dishes were treated with increasing concentration of GRWE for 24 hr. Treated cells were washed twice with ice-cold PBS and DNA was purified according to some modified manufacturer's protocol. DNA was loaded into a 1.2% agarose gel contained 0.5 ㎍/㎖ ethidium bromide at 100 V for 2 hr and visualized under UV light.

6. DNA Content (FACS) analysis

After the appropriate treatment, HL-60 cells were harvested by centrifugation and wahsed with PBS. The cells were fixed with ice-cold 100% ethanol for 60min, washed with PBS, and then treated with 10 ㎍/㎖ propidium iodide (Sigma) /100 ㎍/㎖ RNase A (Sigma) solution at 37℃ for 1 h in the dark. and analyzed on a fluorescence- activated flow cytometer.

7. Subcellular fractionation

Subcellular fractionation was carried out as described by previous report.[31] After GRWE treatment for 24 hr, cells (4×10^6) were harvested, washed once in ice-cold phosphate-buffered saline (PBS), and gently lysed for 30 sec in 80 ㎕ of ice-cold lysis buffer (250mM sucrose, 1mM EDTA, 0.05% digitonin, 25mM Tris-HCl, pH 6.8, 1mM DTT) with freshly added 1% protease inhibitor cocktail (Sigma). Lysates were centrifuged at 11,000 rpm at 4℃ at 4 min. to obtain the supernantants (cytosolic fraction free of mitochondria) and the pellet (the fraction that contains mitochondria). Supernatants and pellets were electrophoresed on a 15% polyacrylamide gel and then analyzed by Western blot using mouse monoclonal anti-human anti-cytochrome c (BD Pharmingen) and rabbit polyclonal anti-human anti-Bax (Santa Cruz) antibodies and SuperSignal West Pico Chemiluminescent (PIERCE).

8. Western blot analysis

Cells were washed with ice-cold PBS and gently resuspend in ice-cold lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Triton X-100) with freshly added 1% protease inhibitor cocktail and incubate on ice for 30 min. Cell lysates were centrifuged at 14,000 rpm for 10min at 4℃, and the protein concentration was determined using a Bradford assay. After SDS-PAGE, proteins were transferred to nitorcellulose (Schleicher&Schuell) for 3 hr at 40V. Blot were probed with mouse monoclonal anti-human anti-PARP, anti-Bid (BD Pharmingen), and rabbit polyclonal anti-human anti-caspase-3, anti-caspase-8 (Santa Cruz) antibodies. Immunoreactivity was detected using either and anti-mouse (Sigma) or anti-rabbit (Zymed) peroxidase-conjugated sencondary immunoglobulin G antibody followed by SuperSignal West Pico Chemiluminescent (PIERCE).

Results

1. Cytotoxic effect of GRWE in lymphoma and leukemia cell lines

The effect of GRWE on the cytotoxicity of cells was evaluated using the MTT assay. Shown as Fig. 1, a 24 h exposure to GRWE dramatically decreased the viability of HL-60 cells in a concentration-dependent manner, but other cell lines (U-937, Jurkat, Molt-4) was not represent almost cytotoxicity. The concentration required to inhibit growth of HL-60 cells by 50% (IC_{50}) was approximately 100 ㎍/㎖.

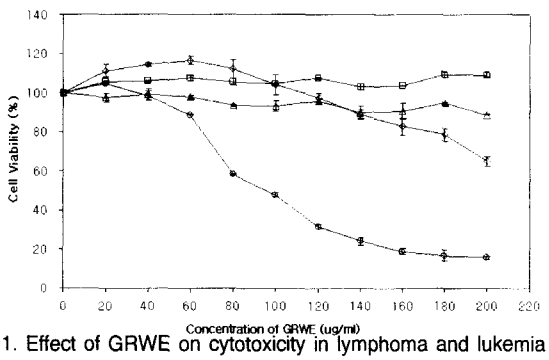

Fig. 1. Effect of GRWE on cytotoxicity in lymphoma and lukemia cell lines. The cells (2×10^5 cells/㎖) were treated with various concentrations of GRWE and the cells were tested for viability by MTT assay 24 h after the treatment of GRWE. U-937 (□), Jurkat (◇), Molt-4 (△) and HL-60 cells (○).

2. Morphological changes in HL-60 cells treated with GRWE

Morphological characteristics of apoptosis, including nuclear condensation and fragmentation, were observed under fluorescent microscope by DAPI staining (Fig. 2). Along with the appearence of elongated cells, disintegrated cells, as evidenced by apoptotic bodies, and cells with condensed nuclear chromatin appeared in a concentration-dependent manner in response to GRWE treatment.

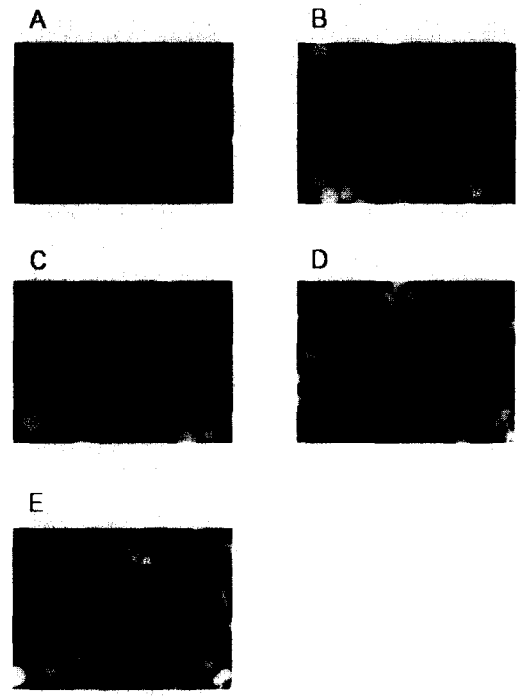

Fig. 2. Effect of GRWE on DNA fragmentation in HL-60 cells. A dose-dependent of DNA fragmentation. HL-60 cells were exposed to the indicated concentration of GRWE for 24 h. DNA was extracted, then separated by 1.2% agarose gel contained ethidium bromide electrophoresis, and visualized under UV light. M, 123bp DNA ladder marker; lane 1, control; lane 2, 25 ㎍/㎖ of GRWE; lane 3, 50 ㎍/㎖; lane 4, 100 ㎍/㎖; lane 5, 200 ㎍/㎖.

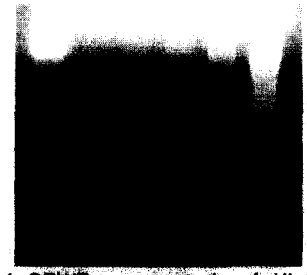

Fig. 3. Effect of GRWE on apoptosis of HL-60 cells by flow cytometric analysis. Flow cytometric analysis of DNA fragmentation following GRWE treatment. HL-60 cells were treated with various concentrations (20~120 ㎍/㎖) of GRWE for 24 h. The harvested cells were fixed in fixed 100% ethanol and stained with propidium iodide, followed by flow cytometric analysis. The percentages of cells with hypodiploid DNA content represent fractions undergoing apoptotic DNA degradation. (A) control; (B) 10 ㎍/㎖ of GRWE; (C) 50 ㎍/㎖; (D) 100 ㎍/㎖; (E) 200 ㎍/㎖.

3. GRWE 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. A ladder pattern of internucleosomal DNA fragmentation was observed when 80 and 120 ㎍/㎖, of GRWE was applied to the HL-60 cells (Fig. 3). To further determine the degree of

apoptosis, we employed flow cytometry to quantify the sub-G1 peak (apoptotic peak). Fig. 4. shows that GRWE increased apoptotic peak in 80 and 120 ㎍/㎖. These results suggest that GRWE can trigger apoptosis of HL-60 cells.

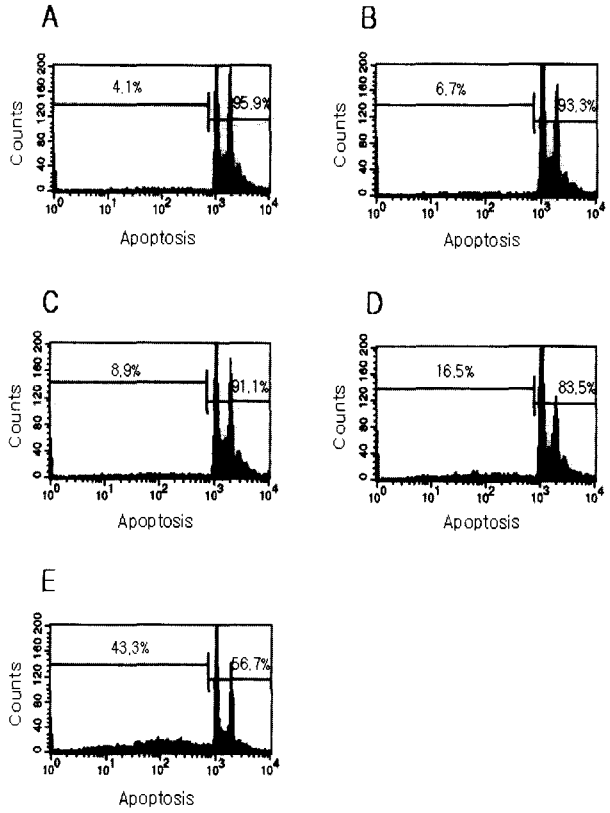

Fig. 4. Effect of GRWE on HL-60 cells morphology. HL-60 cells (2×10^5 cells/㎖) were treated with various concentrations (20~120 ㎍/㎖) of GRWE for 24 h. After, cells were stained with DAPI, subjected to cytospin, and observed under a fluorescence microscope. (A) control; (B) 10 ㎍/㎖ of GRWE ; (C) 50 ㎍/㎖; (D) 100 ㎍/㎖; (E) 200 ㎍/㎖.

4. Effect of GRWE on cell cycle progression of HL-60

Treatment of HL-60 cells with increasing concentrations of GRWE for 24 h led to profound changes of the cell cycle profiles (Fig. 5). The HL-60 cells had a decreased number of cells (23%) in the G1 phase of the cell cycle after their exposure to GRWE (120 ㎍/㎖) as compared with non-treated HL-60 cells (41% in G1). Together, these data suggest that GRWE results in a G1 arrest in cell cycle progression of HL-60 cells.

5. GRWE induces activation of caspase-3 and -8

Caspase family plays key roles in the execution of apoptotic cell death. Of these, caspase-3 and -8 is the most important cell executioners for apoptosis. We observed the proteolytic activation of procaspase-3 and -8 induced by the GRWE by Western blot analysis. At 40 ㎍/㎖, GRWE induced a nearly quantitative cleavage of procaspase-3 (Fig 6A). The cleavage of procaspase-3 was in a concentration-dependent manner. Activation of caspase-3 lead to the cleavage of a number of proteins, one of which is poly(ADP-ribose) polymerase (PARP). Although PARP is not essential for cell viability, the cleavage of PARP is another hallmark of apoptosis. Treatment of HL-60 cells with various concentrations of GRWE induced proteolytic cleavage of 116 kDa PARP with accumulation of the 89 kDa cleaved products. Procaspase-8 was also activated by treatment with GRWE. As shown in Fig. 6B. procaspase-8 was cleavaged from 20 ㎍/㎖. These data suggest that GRWE induce apoptotic cell death through caspase-8 activation.

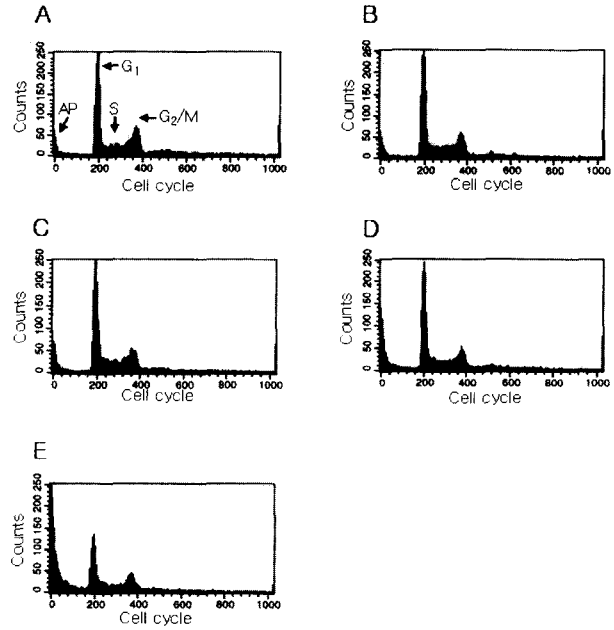

Fig. 5. Cell cycle analysis of HL-60 cells treated with GRWE . HL-60 cells (2×10^5 cells/㎖) were treated with various concentrations (20~120 ㎍/㎖) of GRWE for 24 h. The cells were harvested, and DNA content was analyzed by FACS after propidium iodide staining of RNase-digested, fixed cells. (A) control; (B) 10 ㎍/㎖ of GRWE ; (C) 50 ㎍/㎖; (D) 100 ㎍/㎖; (E) 200 ㎍/㎖.

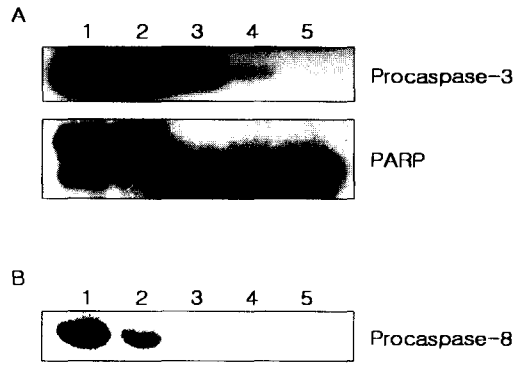

Fig. 6. Effect of GRWE on processing of procaspase-3, -8 and proteolytic cleavage of PARP in HL-60 cells. The cells were treated with GRWE at the indicated concentration for 24 h. Whole cell lysates were subjected to SDS-PAGE followed by blotting with an anti-caspase-3, anti-caspase-8 polyclonal, and anti-PARP monoclonal antibodies. (A) Processing of procaspase-3 and Cleavage of PARP. (B) Processing of procaspase 8. Lane 1, control; lane 2, 10 ㎍/㎖ of GRWE ; lane 3, 50 ㎍/㎖; lane 4, 100 ㎍/㎖; lane 5, 200 ㎍/㎖.

6. GRWE induces mitochondrial cytochrome c release.

To analyze the involvement of cytochrome c release in GRWE-induced apoptosis, cytosolic and mitochondrial fractions were obteined and analyzed for the presence of cytochrome c by Western blot analysis. As shown in Fig. 7, GRWE induced the release of cytochrome c in the cytosolic fractions of HL-60 cells from 40 ㎍/㎖. Also, GRWE-induced cytochrome c release was observed after caspase-8 activation. This is reflected that release of cytochrome c mediates by caspase-8.

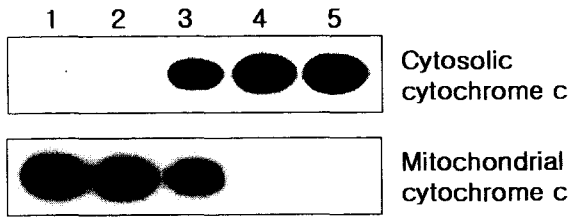

Fig. 7. Effect of GRWE on cytochrome c release in HL-60 cells. Cells treated with different concentration of GRWE for 24 h. Cytosolic and mitochondrial fractions were isolated as described under Materials and methods. Cytochrome c was determined by western blot using anti-cytochrome c monoclonal antibody. Lane 1, control; lane 2, 10 ㎍/㎖ of GRWE ; lane 3, 50 ㎍/㎖; lane 4, 100 ㎍/㎖; lane 5, 200 ㎍/㎖.

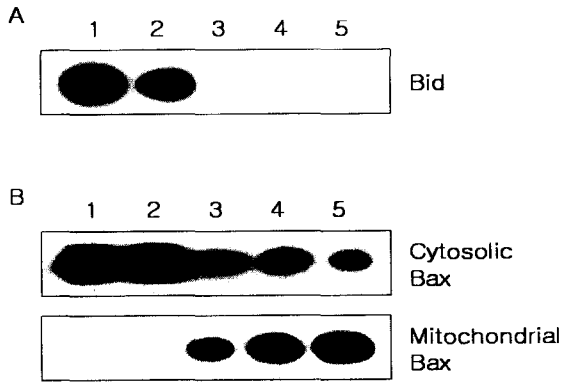

Fig. 8. Effect of GRWE on cleavage of Bid and translocation of Bax in HL-60 cells. Western blot analysis of Bid cleavage and Bax translocation in HL-60 cells treated with GRWE at the indicated concentration for 24 h. Bid and Bax was determined by using anti-Bid monclonal and anti-Bax polyclonal antibodies receptively. (A) Cleavage of Bid. (B) Translocation of Bax to mitochondria. Lane 1, control; lane 2, 10 ㎍/㎖ of GRWE ; lane 2, 50 ㎍/㎖; lane 4, 100 ㎍/㎖; lane 5, 200 ㎍/㎖.

7. GRWE-induced cytochrome c release through Bid cleavage and Bax translocation.

The proapoptotic members of Bcl-2 family such as Bid and Bax play key roles in many drug-induced cytochrome c release. Induction of cytochrome c release from mitochondria occurs via caspase-8-mediated cleavage of Bid or Bax mitochondrial translocation. Also, Bid is one of the triggers for Bax translocation to mitochondria[29,32-35]. We analyzed Bid cleavage and Bax translocation by Western blot analysis. As shown in Fig. 8A, cleavage of Bid was observed from 20 ㎍/㎖ and this corresponded with caspase-8 activation pattern. Bax translocation was also observed from 40 ㎍/㎖ (Fig 8B). These data indicate that GRWE depolarizes the potential of mitochondrial membranes via truncation of Bid, which stimulates the translocation of Bax to mitochondria.

Discussion

GRWE has been found to have antitumor activity. But that did not showed any cytotoxicity at some kind of cancer cell lines. Only leukemia cell lines (Jurkat, Molt-4, HL-60) showed various cytotoxicity (data not shown). Above all things, GRWE showed cell-specifically cytotoxic to human promyelocytic leukemia (HL-60) cells (Fig. 2). These results indicate that GRWE may have a possibility of potential chemotherapeutic agent for human promyelocytic leukemia disease.

In present study. we have shown that GRWE was cytotoxic to HL-60 cells; the IC_{50} value was approximately 100 ㎍/㎖. and GRWE -induced cell death via an apoptotic pathway in HL-60 cells. GRWE was also shown cell-specific cytotoxicity. Analysis of cell morphology, DNA fragmentation, and FACS in HL-60 cells incubated with GRWE suggests that the cytotoxicity of GRWE was mediated by the induction of apoptosis.

Flow cytometric analysis revealed that GRWE -treated HL-60 cells were arrested in the G1 phase. Also, these data shown that the preponderance of apoptotic nuclei was most likely blocked at the G1 phase. Apoptosis and proliferation are linked by cell cycle regulators, and apoptotic stimuli affect both cell proliferation and death,[36] Cell cycle components such as p53, pRb and E2F, have been shown to participate in both cell cycle progression and apoptosis.[37,38] p53, pRb, and E2F have also been acted as G1 regulators. p53 and E2F are involved in the elimination of abnormal cells through apoptosis. In constrast pRb induces G1 arrest and suppresses apoptosis. We well be examine mechnism for cell cycle arrest by GRWE through futher studies of common components of apoptotic and cell cycle machinery.

Recently, a variety of molecules participating in the biochemical pathway that mediates the process of apoptosis have been identified[39]. The signaling pathway of apoptotic cell death is not single, but diverse: there have been at least 3 known major pathways including the mitochondria-bypass pathway as mentioned in the introduction of this report. In the present report, we describe the mechanism through whic GRWE induces apoptosis of HL-60 cells. GRWE activated caspase-8, induced Bid cleavage and Bax translocation, caused mitochondrial cytochrome c release, and induced caspase-3

activation and PARP cleavage in HL-60 cells but not in Bcl-2 (data not shown)

Typically two different pathways leading to apoptosis via caspase-8 have been identified, namely receptor-mediated and non-receptor-mediated apoptosis.[17,40] Most cytokines (usually members of the TNF superfamily) induce apoptosis by interaction of the ligand with its death receptor, which sequentially recruits TNF receptor-associated death domain; Fas-associated death domain (FADD); FADD-like interleukin-1 converting enzyme (FLICE) (also called caspase-8), and caspase-3; the last then cleaves various substrates leading to apoptosis. In contrast, non-receptor (most chemotherapeutic agents)-mediated apoptosis involves cleavage of Bid by caspase-8, which causes the release of cytochrome c from the mitochondria, and cytochrome c together with Apaf1 activates caspase-9, and the latter then activates caspase-3, resulting in apoptosis.

This is the first report to suggest that GRWE activates caspase-8 and that it is required for apoptosis induced by GRWE , but how GRWE activates caspase-8 is not clear. Because autoactivation induced by oligomerization can activate caspase-8[41,42], GRWE may induce oligomerization of caspase-8. However, certain chemotherapeutic agents are known to induce apoptosis through induction of death receptors[43]. We are need futher studies for caspase-8 activation by GRWE in HL-60 cells.

Also, GRWE can induce cleavage of the proapototic protein Bid. Bid exists in the cytosolic fraction of living cells as an inactive precursor that becomes activated upon cleavage by caspase-8. After cleavage, Bid translocates onto mitochondria. The actived Bid by itself is sufficient to induce complete release of cytochrome c from mitochondria. In this study we have shown that Bax mediates mitochondrial cytochrome c release during LKJ2-induced apoptosis. Bax is another proapoptotic member of the Bcl-2 family of proteins. Bax translocates from its predominantly cytoplasmic location to the mitochondria upon apoptosis induction. However, Bid is much more potent cytochrome c releasing factor than Bax. Bax only release up to 20% of the total mitochondrial cytochrome c even at a high concentration[44]. Also, cleaved Bid is reported to be more efficient for triggering the oligomerization and translocation of Bax into mitochondrial membrane[29,32-35].

Conclusion

In conculsion, GRWE mediates apoptosis of HL-60 cells through caspase-8 which involves cleavage of Bid and translocation of Bax to mitochondria where it promotes the release of cytochrome c. Our results contribute to the ordering of events druing GRWE-induced apoptosis, by demonstrating that caspase-8 is responsible for cytochrome c release and caspase-3 activation. Further studies will be required to identify the specific signals that induce caspase-8 activation by GRWE.

Acknowledgement

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