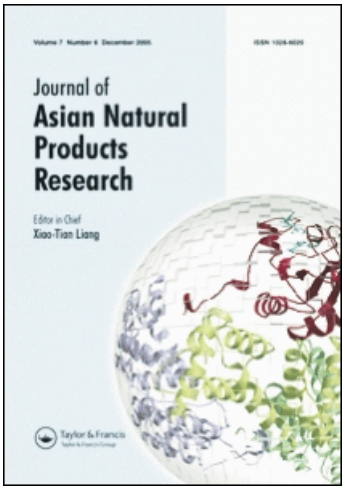

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Diosgenin-3-*O*-α-l-Rhamnopyranosyl-(1→4)-β-d-glucopyranoside obtained as a new anticancer agent from *Dioscorea futschauensis* induces apoptosis on human colon carcinoma HCT-15 cells *via* mitochondria-controlled apoptotic pathway

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DIOSGENIN-3-*O*-α-L-RHAMNOPYRANOSYL-(1→4)-β-D-GLUCOPYRANOSIDE OBTAINED AS A NEW ANTICANCER AGENT FROM *DIOSCOREA FUTSCHAUENSIS* INDUCES APOPTOSIS ON HUMAN COLON CARCINOMA HCT-15 CELLS *VIA* MITOCHONDRIA-CONTROLLED APOPTOTIC PATHWAY

SAN-LONG WANG[a,b], BING CAI[a,*], CHENG-BIN CUI[a,c,†], HONG-WEI LIU[b], CHUN-FU WU[b] and XIN-SHENG YAO[b,d]

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Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (DRG) is a well-known pentacyclic triterpene glycoside newly isolated from the rhizomes of *Dioscorea futschauensis* R. Kunth (Dioscoreaceae) by our group. In the present work, the inhibitory effect of DRG on the cell proliferation of human cancer cell lines was examined to reveal for the first time that DRG shows stronger anticancer activity than that of the positive control cisplatin. DRG inhibited the proliferation of human cancer cells, A431, A2780, A549, K562, and HCT-15, with IC_{50} ($\mu mol\,L^{-1}$) values of 9.33 ± 0.22, 18.7 ± 0.16, 9.98 ± 0.38, 6.44 ± 0.10, and 5.86 ± 0.14 respectively. It was then found, by morphological observation, "DNA ladder" detection and flow cytometric analysis, that DRG exerts its anticancer effect through inducing apoptosis on HCT-15 cells. Furthermore, it has been demonstrated that DRG triggers a mitochondria-controlled apoptotic pathway to induce apoptosis on HCT-15 cells, which involves the reduction of the mitochondrial potential ($\Delta\psi_m$), the release of cytochrome *c* from mitochondria into the cytosol, and the down-regulation of the ratio of Bcl-2/Bax expression level. The present results reasonably suggest that regulating the balance of Bcl-2/Bax expression level plays a key role in the DRG-induced apoptosis. Such findings provide novel knowledge to elucidate the biological properties of DRG, even though DRG was discovered early in the late 1960s. These results suggest that DRG may be a good candidate as a chemotherapeutic agent to treat human colon carcinoma.

Keywords: Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside; Apoptosis; Bax; Bcl-2; Cytochrome *c*; Mitochondrial transmembrane potential; HCT-15 cells; *Dioscorea futschauensis*

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INTRODUCTION

Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (DRG) (Fig. 1), a pentacyclic triterpene glycoside once called prosapogenin B of dioscin, was isolated from *Dioscore tokoro* by Kawasaki *et al.* for the first time in 1968 [1]. Later Seshadri *et al.* and Espejo *et al.* also obtained the same compound, respectively, from *Paris polyphylla* in 1972 [2] and from the fermented material of *Dioscorea composita* in 1982 [3]. In our recent studies on bioactive substances from natural resources, we isolated this compound from *Dioscorea futschauensis* R. Kunth (Dioscoreaceae), the source plant of the traditional Chinese medicine "Mian Bi Xie", which is used to treat rheumatism and urinary tract disease [4] (Fig. 1).

Even though DRG is well known, no report had been made on its anticancer activity. In the present study, we have found, for the first time, that DRG could inhibit significantly the cell proliferation of several human cancer cell lines, A2780 (human ovarian adenocarcinoma), A431 (human epidermoid carcinoma), A549 (human lung adenocarcinoma), HCT-15 (human colon carcinoma) and K562 (human erythroleukemia). We have also demonstrated that DRG can induce apoptosis on HCT-15 cells *via* a mitochondria-controlled apoptotic pathway involving the reduction of mitochondrial membrane potential, the induction of cytochrome *c* release into the cytosol, and the down-regulation of the ratio of Bcl-2/Bax expression level, which should be the origin of the cytochrome *c* release to induce apoptosis.

RESULTS AND DISCUSSION

DRG Significantly Inhibits the Proliferation of Human Cancer Cells

The effects of DRG on the proliferation of the human A2780, A431, A549, HCT-15 and K562 cancer cell lines were assayed by MTT method. As summarized in Table I, DRG inhibited the proliferation of both solid-tumor (A2780, A431, A549 and HCT-15) and nonsolid-tumor (K562) cells, and its inhibitory effect was stronger than that of the cisplatin (CDDP) used as the positive control. In addition, the HCT-15 cells were more sensitive to DRG than the other cells tested. Thus further studies on the effects of DRG were carried out using HCT-15 cells.

Evidences for Apoptosis Induced by DRG to Inhibit Cancer Cell Proliferation

Morphology

The treatment of HCT-15 cells with DRG at different concentrations for different time periods caused significant changes on the cell morphology observed under the light microscope, exhibiting typical features of apoptosis such as cell shrinkage, membrane blebbing and formation of membrane-enclosed vesicles (apoptotic bodies) [5].

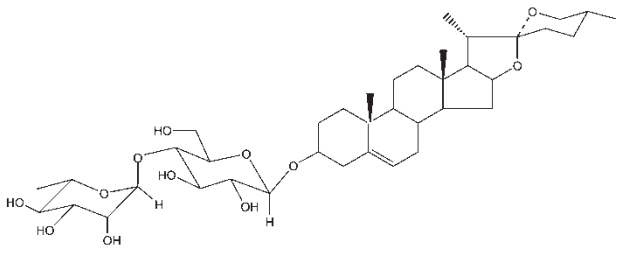

FIGURE 1 Structure of DRG.

TABLE I Inhibitory effect of DRG and CDDP on proliferation of human cancer cells

	IC_{50} ($\mu mol\,L^{-1}$) in the indicated cell lines[a]				
Compounds	*A431*	*A2780*	*A549*	*K562*	*HCT-15*
DRG	9.33 ± 0.22	18.7 ± 0.16	9.98 ± 0.38	6.44 ± 0.10	5.86 ± 0.14
CDDP	17.2 ± 0.12	> 100	> 100	50.0 ± 0.15	49.5 ± 0.11

[a] IC_{50} is defined as the concentration that results in a 50% decrease of viable cell numbers. The data represent mean values of three independent experiments, which were determined by the MTT method.

Typical morphological observations for the HCT-15 cells are shown in Fig. 2 (A and B), recorded after exposing the cells to 10 μmol L^{-1} DRG for 24 h. Correspondingly, nuclei of the cells visualized by fluorescent staining with Hoechst 33258 reagent showed morphological characteristics of chromatin condensation, as shown in Fig. 2 (C and D), and the morphology of cell nuclei directly observed under transmission electron microscope also gave characteristics typical of chromatin condensation, cytoplasmic and nuclear membrane blebbing and the formation of nuclear membrane-enclosed vesicles (Fig. 2 E and F), which are typical of apoptotic cell nuclei. These observations suggested that DRG induced apoptosis on HCT-15 cells to inhibit the cell proliferation.

Nucleosomal DNA Ladder

A characteristic biochemical feature of the apoptosis is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes, leading to the production of oligonucleosomal fragments. Internucleosomal DNA fragmentation forms integer multiples of 180–200 bp fragments. This kind of degradation pattern can be detected as a "nucleosomal DNA ladder" by agarose gel electrophoresis [5]. Analyses of DNA samples from HCT-15 cells treated with 10 μmol L^{-1} DRG and 50 μmol L^{-1} CDDP for 24 h clearly showed the "nucleosomal DNA ladder" for both samples by agarose gel electrophoresis (Fig. 3). Upon increasing the treatment times, the "DNA ladder" was detected more clearly, evidencing a time-dependent inducement of apoptosis by DRG on HCT-15 cells [Fig. 3(b)].

Flow Cytometric Analysis

The induction of apoptosis by DRG was also evidenced by flow cytometric analysis of the HCT-15 cells treated with DRG. Nuclei that appeared in the sub-G_0/G_1 peak region on the flow cytometric histogram were considered as apoptotic, containing hypodiploid DNA. As shown in Fig. 4, treatment of the HCT-15 cells with 10 μmol L^{-1} DRG for 24 h remarkably increased the percentage (46.7%) of apoptotic cells over that (2.4%) of the control, while a 50 μmol L^{-1} CDDP treatment only caused the appearance of 37.3% apoptotic cells, indicating that the effect of DRG was stronger than that of CDDP.

DRG Triggers a Mitochondria-controlled Apoptotic Pathway

Studies have demonstrated that mitochondria play a key role in the progression of apoptosis [6,7]. A crucial and common step in apoptosis is postulated to involve loss of mitochondrial membrane potential ($\Delta\psi_m$) [6]. This potential is essential for various cellular functions, including the production of ATP *via* oxidative phosphorylation [8]. Conversely, induction of apoptotic cell death is associated with the mictochondrial release of cytochrome *c* [9,10] and the leakage of cytochrome *c* has been considered to result from the opening of a membrane

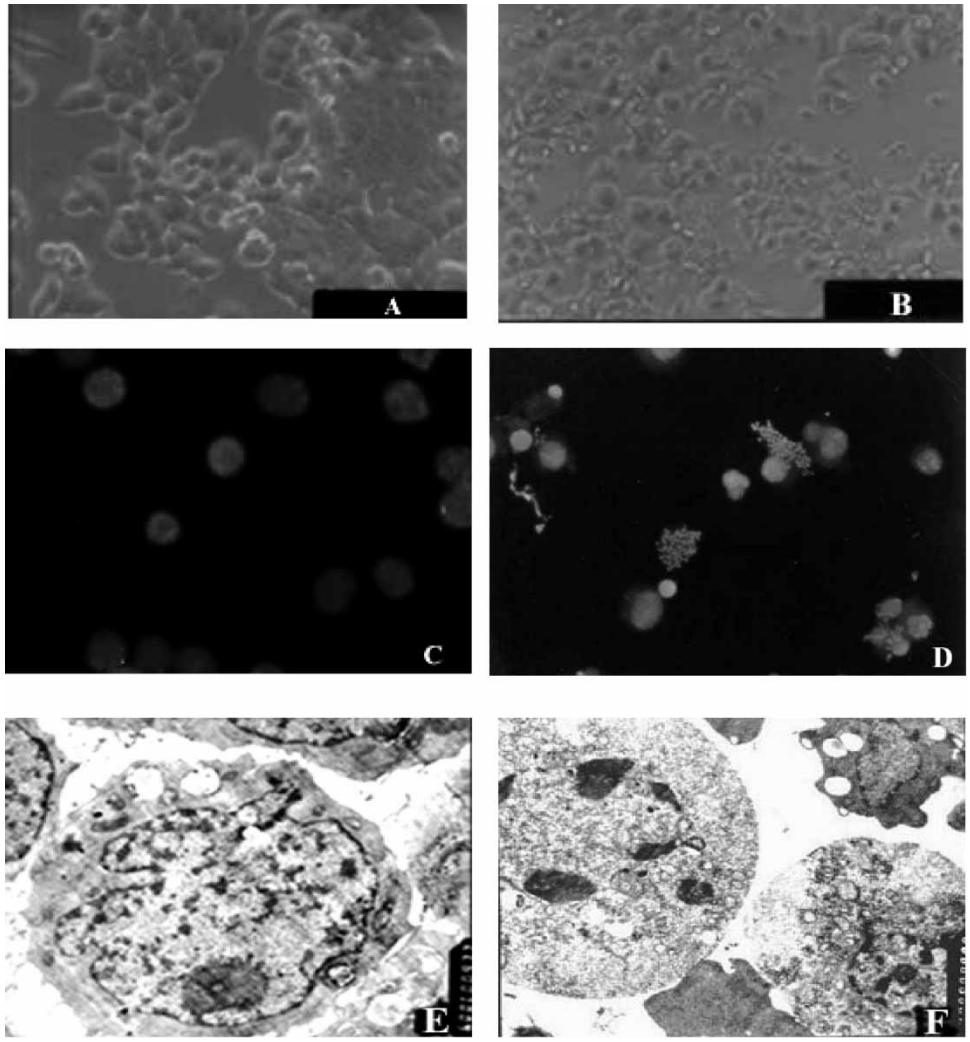

FIGURE 2 Microscope observations of the HCT-15 cells treated with DRG. (A) and (B): Photos taken under light microscope, (A) negative control, (B) cells treated with 10 μmol L^{-1} DRG for 24 h. (C) and (D): Photos taken under fluorescence microscope after staining the cell nuclei with the Hoechst 33258 reagent, (C) negative control, (D) cells treated with 10 μmol L^{-1} DRG for 24 h. (E) and (F): Photos taken under transmission electron microscope, (E) negative control, (F) cells treated with 10 μmol L^{-1} DRG for 24 h.

permeability pore and the loss of the $\Delta\psi_m$ [11]. To investigate the involvement and temporal relationship of mitochondrial events in DRG-induced apoptosis, the HCT-15 cells were stimulated with DRG and then the translocation of cytochrome *c* into the cytosol and the reduction of $\Delta\psi_m$ were analyzed in a time course experiment.

Reduction of Mitochondrial Transmembrane Potential ($\Delta\psi_m$)

The $\Delta\psi_m$ was measured by using rhodamine 123, a cell permeable, cationic, fluorescent dye that can incorporate into mitochondria depending on the inner transmembrane potential. The declining uptake of rhodamine 123 reflects a reduction of $\Delta\psi_m$, which is a signal for the opening of a mitochondrial megachannel that is also called the permeability transition pore [12].

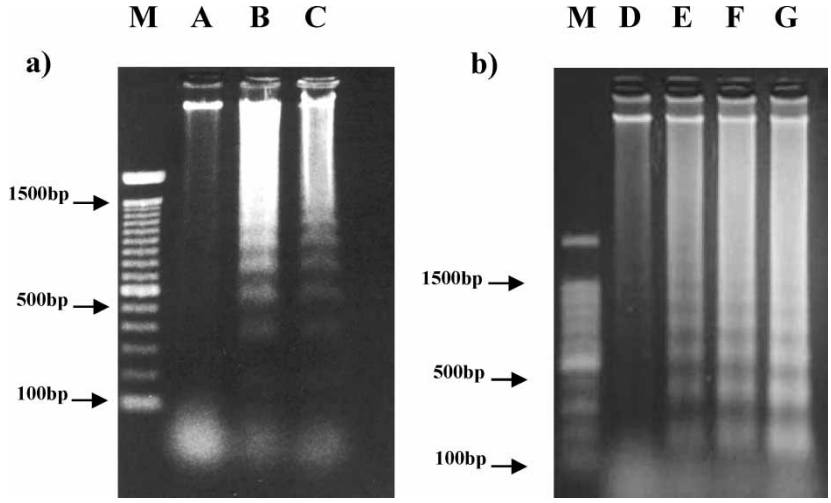

FIGURE 3 DNA ladder detected by agarose gel electrophoresis. DNA was extracted from the HCT-15 cells treated with the samples for 6, 12 or 24 h and analyzed by 1.5% agarose gel electrophoresis at 40 V: (a) M 100 bp DNA marker; (A) negative control; (B) 50 μmol L^{-1} CDDP for 24 h; (C) 10 μmol L^{-1} DRG for 24 h; (b) M 100 bp DNA marker; (D) negative control; 10 μmol L^{-1} DRG for (E) 6 h, (F) 12 h and (G) 24 h.

Following treatment of the HCT-15 cells with DRG (10 μmol L^{-1}) for 0, 6, 12 and 24 h, the $\Delta\psi_m$ of the cells was lost in a time-dependent manner, beginning as early as 6 h after exposure of HCT-15 cells to DRG (Fig. 5). The $\Delta\psi_m$ decreased with exposure time up to 24 h. Thus, the DRG-induced apoptosis was considered to accompany with drop of $\Delta\psi_m$.

Release of Cytochrome c from Mitochondria into the Cytosol

The cytochrome *c* release was measured by 15% SDS-PAGE loading cytosolic fractions obtained after depleting mitochondria from the DRG-treated HCT-15 cells. As shown in Fig. 6, the DRG-treatment (10 μmol L^{-1}) for 6, 12 and 24 h resulted in the release of cytochrome *c* in a time-dependent manner in the HCT-15 cells, and the cytochrome *c* release correlated well with the reduction of $\Delta\psi_m$.

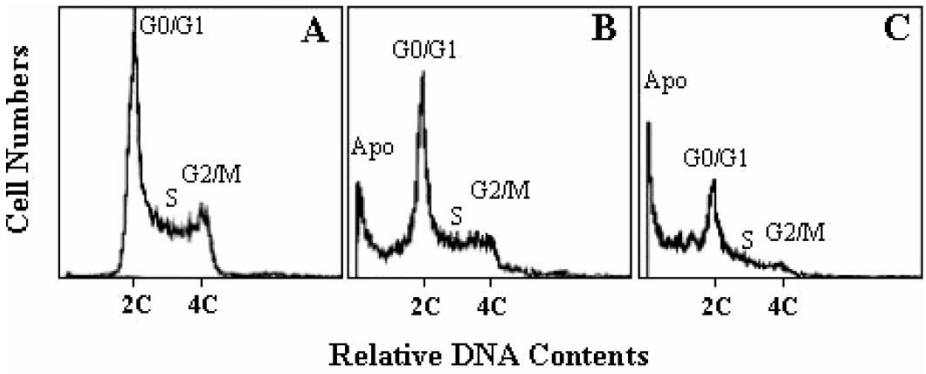

FIGURE 4 Flow cytometric histograms of the HCT-15 cells. The HCT-15 cells treated with the samples for 24 h were fixed in ice-cold ethanol, stained with propidium iodide and then subjected to the flow cytometric analysis. (A) Negative control; (B) 50 μmol L^{-1} CDDP; (C) 10 μmol L^{-1} DRG.

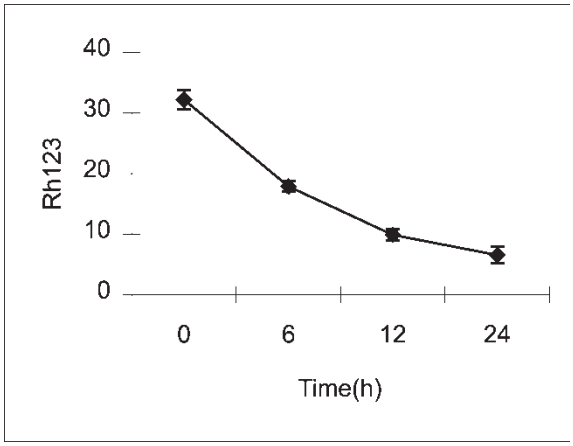

FIGURE 5 Effect of DRG on mitochondrial membrane potential ($\Delta\psi_m$) in HCT-15 cells. The HCT-15 cells treated with 10 μmol L^{-1} DRG for 0, 6, 12 and 24 h were collected at the indicated time point, stained with rhodamine 123 and then analyzed by flow cytometry. The intensity of fluorescence (Rh123) reflects the relative mitochondrial membrane potential ($\Delta\psi_m$). The data each represent the mean value of three independent experiments.

Down-regulation of the Ratio of Bcl-2/Bax Expression Level

It has been shown that Bax and Bcl-2 proteins regulate apoptosis mainly *via* the mitochondria-controlled pathway [6–15]. A key step of this pathway is the release of cytochrome *c* into cytoplasm, where the cytochrome *c* was activated, and then the caspases crucial for the execution of apoptosis are activated. Bcl-2 proteins interfere with the activation of caspases by preventing the release of cytochrome *c*, while Bax proteins induce the cytochrome *c* release [13,14]. To investigate the biochemical mechanism of DRG-induced apoptosis, the expression of Bcl-2/Bax proteins in the HCT-15 cells treated with DRG were examined by Western blot analysis in a time course experiment.

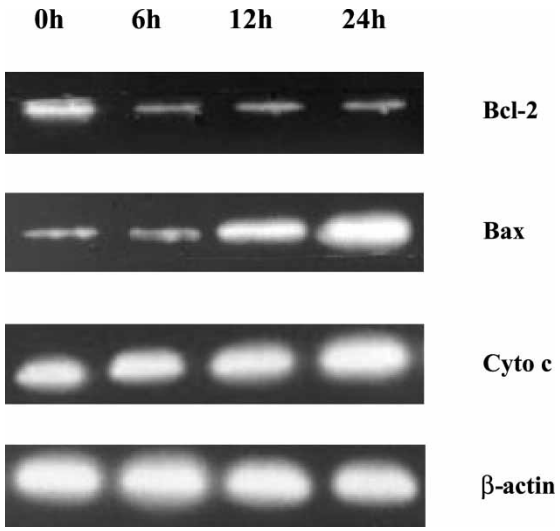

FIGURE 6 Western blot analysis. The proteins were extracted from the HCT-15 cells treated with 10 μmol L^{-1} DRG for 0, 6, 12 or 24 h, respectively, and separated with 12–15% SDS-PAGE. The proteins, Bcl-2, Bax, cytochrome *c*, and β-actin were detected by immunoblotting using specific antibodies. The β-actin was used as a control.

As shown in Fig. 6, the DRG-treatment suppressed significantly the expression of Bcl-2 from 6 h, which is consistent with the initial reduction of $\Delta\psi_m$ (Fig. 5) and cytochrome *c* release (Fig. 6), while the DRG-treatment caused the overexpression of Bax from 12 h, which largely lagged the initial reduction of $\Delta\psi_m$ (Fig. 5) and cytochrome *c* release (Fig. 6). As a whole, the ratio of Bcl-2/Bax expression level was down-regulated by the DRG-treatment in the HCT-15 cells, which seems to result in cytochrome *c* release to induce apoptosis. The expression of β-actin, however, was not affected by DRG-treatment (Fig. 6).

Concluding Remarks

In the present work, the inhibitory effect of DRG on the cell proliferation of human cancer cell lines A431, A2780, A549, K562 and HCT-15 was examined to reveal for the first time that DRG shows stronger anticancer activity than that of the positive control CDDP. Subsequent morphological observation under light, fluorescent and transmission electron microscopes, "DNA ladder" detection using agarose gel electrophoresis and flow cytometric analysis showed that DRG exerts its anticancer effect through inducing apoptosis on HCT-15 cells. Furthermore, it has been demonstrated that DRG triggers a mitochondria-controlled apoptotic pathway to induce apoptosis on HCT-15 cells, which involves the reduction of mitochondrial potential ($\Delta\psi_m$), the release of cytochrome *c* from mitochondria into the cytosol, and the down-regulation of the ratio of the Bcl-2/Bax expression level.

Furthermore, the present results demonstrated that the time-dependent reduction of $\Delta\psi_m$ (Fig. 5) correlated well with the cytochrome *c* release from the mitochondria into the cytosol in the DRG-induced apoptosis, and that the release of cytochrome *c* was coincident with the down-regulation of the ratio of the Bcl-2/Bax expression level (Fig. 6). In addition, the release of cytochrome *c* and the reduction of $\Delta\psi_m$ in the DRG-induced apoptosis were initiated during the early stages of DRG-treatment (6 h), from which stage the expression of Bcl-2 has been suppressed to a lower level while the expression of Bax was not affected at that time period (see Fig. 6). From the 12 h point of DRG-treatment, the expression level of Bcl-2 was not changed by comparison with that at 6 h, while the expression of Bax began to increase significantly in a time-dependent manner as shown in Fig. 6. Corresponding with the increase of Bax expression level, the release of cytochrome *c* and the reduction of $\Delta\psi_m$ increased more remarkably in a time-dependent manner in the DRG-induced apoptosis.

The Bcl-2 family members such as Bcl-2, Bcl-xL and Bax localize to the outer mitochondrial membrane. Bax is found as intracellular membrane-associated proteins of the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope [15]. Oligomeric Bax forms one of the ion channels in synthetic lipid membranes and the role of Bax as a pro-apoptotic protein appears to be elicited through an intrinsic pore-forming activity. Thus, overexpression of Bax leads to the release of cytochrome *c* from the mitochondrial intermembrane into the cytosol where the cytochrome *c* initiates the activation of a caspase cascade to execute apoptosis [14]. On the other hand, Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria and the overexpression of Bcl-2 prevents the efflux of cytochrome *c* from mitochondria into cytosol to prevent the initiation of apoptosis [9,10].

Although the detailed regulating mechanism for the cytochrome *c* release from mitochondria by Bcl-2/Bax proteins is unknown, our present result suggest that at an early stage of the DRG-treatment, DRG suppressed the expression of Bcl-2 to initiate a limited release of cytochrome *c* by reducing the effect of Bcl-2 on preventing the release of cytochrome *c*, while at the later stage of treatment, from 12 h, the Bax protein was overexpressed dramatically in a time-dependent manner, which, in addition to the effect of

reducing Bcl-2, resulted in the correspondingly dramatic up-regulation of cytochrome *c* release.

The release of cytochrome *c* was well paralleled by the down-regulation of the ratio of Bcl-2/Bax expression level in our time course experiment for the DRG-induced apoptosis, and the $\Delta\psi_m$ loss also paralleled the cytochrome *c* release. These data indicated that down-regulation of the Bcl-2/Bax expression ratio seems to be the origin of cytochrome *c* release, and the release of cytochrome *c* from mitochondria into the cytosol is likely to be the primary cause for the apoptosis induced by DRG, although the relation between the cytochrome *c* release and the loss of $\Delta\psi_m$ is still unclear. Also, it is reasonable to consider from the present result that regulating the balance of the Bcl-2/Bax expression level plays a key role in the induction of the DRG-induced apoptosis. These findings provided novel knowledge for leaning new biological properties of DRG even though it was first found in the 1960s. These results also suggest that DRG may be a good candidate as a chemotherapeutic agent to treat human colon carcinoma.

The detailed molecular mechanism downstream of cytochrome *c* release in the DRG-induced apoptosis is being investigated and will be the subject of a subsequent report.

EXPERIMENTAL

Chemicals and Reagents

DRG was isolated from the rhizomes of *Dioscorea futschauensis* R. Kunth (Dioscoreaceae) by our research group and its purity is over 98% by HPLC analysis.

RPMI-1640 culture medium, penicillin and streptomycin were purchased from GIBCO BRL Laboratories (Grand Island, NY, USA). Fetal bovine serum was the product of Hyclone Co. Hoechst 33258, proteinase K, RNase A, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI) and ethidium bromide (EB) were obtained from Sigma (St. Louis, MO). Rhodamine 123 was the product of Molecular Probes (Eugene, OR). Mouse anti-cytochrome *c* monoclonal antibody (mAb), mouse anti-Bcl-2 mAb, rabbit anti-Bax polyclonal antibody, mouse anti-caspase-3 mAb, mouse anti-PARP (polyADP-ribose polymerase) mAb, mouse anti-human β-actin antibody, and IgG conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

HCT-15 cells were routinely maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum in the presence of 100 units mL^{-1} of penicillin and 100 $\mu g\,mL^{-1}$ of streptomycin and the cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Bioassay for Inhibitory Effect of DRG on Cancer Cell Proliferation

The inhibitory effect of DRG on cancer cell proliferation was assayed by the MTT method [16], a colorimetric assay based on the ability of viable cells to reduce yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to blue formazan. Briefly, exponentially growing cells were suspended in fresh medium at the density of 2×10^5 cells mL^{-1}, seeded onto a 96-well flat microtiter plate and cultured for 24 h in the presence or absence of DRG at various concentrations. Then, 10 μL MTT solutions (5 mg mL^{-1} MTT in PBS filter sterilized) were added into each well and cultured for further

4 h at 37°C. The formazan product was harvested by centrifugation followed by aspirating supernatant and DMSO (100 μL) was added to dissolve it. The optical density (OD) of each well was determined on a SPECTRA MAX Plus plate reader at 570 nm. Data from triple wells were taken for each concentration of samples on each plate and the same experiment was repeated independently at least three times. CDDP was used as a positive control. A negative control group was set using triple wells containing only the cells in drug-free medium and a blank group was set using triple wells containing only the same volume of cell-free medium.

After subtraction of the OD mean value of a blank group to obtain the OD for control (OD_C) and samples (OD_S) at various concentrations, inhibition rates (IR%) for samples at various concentrations were obtained from $IR\% = (OD_C - OD_S)/OD_C$. The concentration required to reduce OD by 50% (IC_{50}) in comparison to control group was then determined.

Microscope Observation

Morphology of the HCT-15 cells cultured for 24 h in the presence or absence of DRG or CDDP in a multi-well plate was directly observed and photographed with an Olympus CK40 inverted system microscope using an Olympus PM-C35B camera system (Olympus, Japan).

Conversely, the cells treated with samples were spread on a glass slide after Carnoy Fixation (methanol–glacial acetic acid 3:1) and the cell nuclei were visualized by fluorescence staining with Hoechst 33258 [17] reagent to examine their nuclear characteristics. An Olympus IX70 photographic system equipped with an Olympus IX70 inverted system microscope, an Olympus IX-FLA inverted reflected light fluorescence observation attachment, a PM-30 automatic photographic system and a JVC TK-C1381 color video camera system were used to observe the nuclear characteristics of the cells and to take photographs.

DNA Extraction and Agarose Gel Electrophoresis

Analysis of internucleosomal DNA fragmentation in HCT-15 cells was performed according to the method of Traganos *et al.* [18]. In brief, cells treated with DRG were collected by centrifugation, washed twice with ice-cold PBS (pH 7.2) and lysed by suspending overnight at 37°C in a lysis-buffer solution of Tris–HCl buffer (100 mmol L^{-1}) (pH 8.5), EDTA (5 mmol L^{-1}), sodium chloride (0.2 mmol L^{-1}), sodium dodecyl sulfate (SDS) [0.2% (W/V)] and proteinase K (200 μL mL^{-1}). Sodium chloride solution (1.5 mol L^{-1}) was then added to the lysate, to give a final sodium chloride concentration of 1 mol L^{-1}, which was centrifuged to obtain a supernatant. To the supernatant ice-cold ethanol was added, to give a final ethanol concentration of 70%, and it was kept at −20°C for 2 h to precipitate DNA. The precipitated DNA obtained by centrifugation was washed twice with 70% ethanol, air dried and dissolved by standing at 37°C for 2 h in a TE buffer containing Tris–HCl buffer (10 mmol L^{-1}) (pH 8.0), EDTA (10 mmol L^{-1}) and DNase-free RNase A (20 μg mL^{-1}). The DNA sample solution was then subjected to electrophoresis at 40 V on a 1.5% agarose gel containing EB (0.5 μg mL^{-1}) and photographed with Polaroid instant pack film T667 under UV light.

Flow Cytometry

The HCT-15 cells treated with samples were harvested, washed twice with ice-cold PBS, and then fixed overnight in 70% ice-cold ethanol. After digestion of RNA by RNase A (25 μg mL^{-1}) for 30 min at 37°C, cells were collected by centrifugation and stained with PI water solution (50 μg mL^{-1} PI, 0.1% sodium citrate and 0.2% Nonidet P-40 in water) for

30 min at 4°C under lightproof conditions. Then the cells (2×10^4) were then collected and analyzed by flow cytometry (Coulter EPICS XL, USA). The distribution of the cells within cell cycle and apoptosis was calculated using the computing program WinCycle (Coulter).

Measurement of Mitochondrial Potential ($\Delta\psi_m$) [19]

The HCT-15 cells, treated with different concentrations of DRG, were incubated for 30 min at 37°C in the presence of the fluorochrome Rhodamine 123 and then detached from dishes by treatment with trypsin. The cells were harvested by centrifugation, washed twice with PBS and then subjected to flow cytometric analysis on the EPICS XL (Coulter). Based on the criteria of acquisition, 2×10^4 cells were collected and analyzed for each sample and the mean value of fluorescence was estimated using CellQuest analysis software (Coulter).

Western Blot Analysis

The HCT-15 cells treated with samples were lysed at 100°C for 5 min in lysis buffer containing NaCl (150 mmol L^{-1}), Tris-HCl (100 mmol L^{-1}) (pH 8.0), EDTA (10 mmol L^{-1}), dithiothreitol (1 mmol L^{-1}), 0.1% Nonidet P-40 and phenylmethylsulfonyl fluoride (100 μg mL^{-1}). The lysate was centrifuged for 10 min at 10,000*g* to remove the particulate materials and the total protein concentration was determined by the bicinchoninic acid (BCA) method using a Pierce BCA Protein Assay Reagent Kit (Pierce, Rockford, IL.). Subsequently, proteins (100 μg per lane) were separated by SDS-PAGE (12% gel for Bcl-2, Bax and β-actin, 15% gel for cytochrome *c*) and electroblotted to a nitrocellulose membrane (Amersham Pharmacia Biotech, Germany). Equal protein loading was controlled by Ponceau-red staining of membranes. Membranes were blocked for 1 h with 4% (w/v) nonfat dry milk powder/0.2% Tween-20 in PBS (PBST) and immunoblotted for 2 h with mouse anti-cytochrome *c* mAb, mouse anti-Bcl-2 mAb, rabbit anti-Bax polyclonal antibody or mouse anti-human β-actin mAb. The membranes were washed 4 × with PBST and incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG, for 2 h at room temperature. After extensive washing, blots were detected by enhanced chemiluminescent staining using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

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