Apoptosis Induced by Dioscin in Hela Cells

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Dioscin, a saponin extracted from the root of Polygonatum Zanlanscianense Pamp, markedly inhibited proliferation of Hela cells. The results indicated that Hela cells underwent apoptosis in dose- and time-dependent manners when treated with Dioscin. Caspase-3, -8 and -9 activities were also detected. The low enzymatic activity of caspase-8 and high activity of caspase-9 showed that the mitochondrial pathway was activated in apoptosis. The reduced expression of the survival protein Bcl-2 also confirmed this result. These studies may be significant in finding a new drug to treat human cervical cancer.

Key words  Dioscin; Hela; apoptosis; caspase; Bcl-2

Many natural products have pharmacological applications, in particular their potential for use in cancer chemoprevention.1—5) Plant products are widely used in testing because of their low toxicity and great medicinal value. Much research has concentrated on different plant extracts’ abilities to induce apoptosis of cancer cells.3—5)

Overwhelming evidence indicates the involvement of caspases in apoptosis. Caspases make up of a family of cysteine proteases that cleave substrates after aspartic residue.6) There are two major apoptotic pathways in mammalian cells. One is the death-receptor pathway and the other is the mitochondrial pathway. Caspase-8 and caspase-9 are activated in these two pathways respectively.7,8) And these two pathways converge at the level of caspase-3 activation.9)

Dioscin, which was extracted from Polygonatum Zanlan-scianense Pamp, has recently been reported to induce differentiation and apoptosis in the human leukemic HL60 cell line.10) This study demonstrated for the first time the apoptotic potential of Dioscin in Hela cells where caspases-9 and caspase-3 were activated, suggesting that Dioscin induced apoptosis of Hela cells through the mitochondrial pathway.

MATERIALS AND METHODS

Chemicals  Dioscin was extracted as previously described.10) RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco Laboratories (NY, U.S.A.). Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma Chemical Co. (MO, U.S.A.). The ApoAlert Annexin V-FITC Apoptosis Kit, the ApoAlert Caspase-3 Fluorescent Assay Kit and the ApoAlert Caspase-8 Colorimetric Assay Kit were purchased from Clontech Laboratories, Inc. (CA, U.S.A.). The Caspase-9/Mch6 Colorimetric Protease Assay Kit was from the Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Bcl-2 monoclonal antibody and FITC-conjugated mouse IgG1 monoclonal isotype control were from BD PharMingen.

Cell Culture  Hela (human cervical cancer cell line) was provided by the Cell Biology Laboratory of Tsinghua University. The cells were cultured in an RPMI-1640 medium (Gibco, NY, U.S.A.) supplemented with 8% fetal bovine serum (Gibco, NY, U.S.A.), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell culture was maintained at 37°C in humidified air with 5% CO2.

Inhibition Effect of Dioscin on Hela Cells  A 200 μl aliquot cell suspension containing 104 cells was added to each well of a 96-well plate. The medium was changed after 24 h, and the cells were then treated with increasing concentrations (1—16 μg) of Dioscin for 48 h at 37°C. After these treatments, 20 μl MTT (5 mg/ml) was added to each well and the cells were incubated for 4 h. Then formazan precipitate was dissolved with 200 μl dimethyl sulfoxide (DMSO). The growth inhibition was determined by measuring the absorption at 550 nm in a microplate reader (Benchmark, Bio-Rad, U.S.A.).

Quantification of Apoptosis by Annexin V/propidium Iodide Staining  Apoptosis in Hela cells was induced with 2, 5 and 8 μg Dioscin for 48 h. Apoptotic cells were determined using an ApoAlert Annexin V-FITC Apoptosis Kit. All the operations were performed according to the user manual. Briefly, 105 cells were rinsed with a binding buffer and were suspended in 200 μl of the same buffer. Then 5 μl of Annexin V (20 μg/ml) and 10 μl of propidium iodide (PI, 50 μg/ml) were added to the suspension. The cells were incubated for 15 min at room temperature in the dark. Then flow cytometric analysis was carried out using a FACScan (Coulter, EPICS ELITE, U.S.A.).

FACS Analysis of Cells in the Sub-G1 Region  FACS analysis was performed for screening cell death, as previously described.11) Cells undergoing apoptosis or necrosis were recognized as an accumulated cell population in the sub-G1 region. After exposure to 5 μg Dioscin for 6, 12, 18, 24, 30, 36, 42 and 48 h, about 106 cells were washed in phosphate buffered saline (PBS) once and then resuspended in 70% ice-cold ethanol for fixing. After treated with RNase A (100 mg/ml) for 30 min at 37°C, DNA was stained with PI (50 μg/ml). The cells were incubated for 15 min at room temperature in the dark. Sub-G1 analysis was performed by FACScan (Coulter, EPICS ELITE, U.S.A.).

Detection of Caspase-3, -8 and -9 Activity from Apoptotic Cells  Hela cells were treated with 2, 5 and 8 μg Dioscin for 24 h. Caspase assay kits were used for testing protease activity. About 2—5×106 cells were gathered and lysed in 50 μl of ice-cold cell lysis buffer. Homogenates were centrifuged at 10000×g for 10 min. Then 50 μl of 2×Reaction Buffer/DTT Mix and 5 μl of caspase-specific substrate (DEVD-AFC for caspase-3, IETD-pNA for caspase-8 and

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LEHD-pNA for caspase-9) were added to the cell lysates. The cleavage of the peptide by caspase released the chromophore. Enzymatic activity of caspase-3 was determined by quantifying fluorescent intensity in a spectrofluorimeter (Hitachi, Japan) with a 400 nm excitation filter and 501.5 nm emission filter using a 100 μl quartz micro-cuvette. Activity of caspase-8 and -9 was determined spectrophotometrically at 405 nm in a microplate reader (Benchmark, Bio-Rad, U.S.A.).

**Intracellular Staining of Bcl-2** Hela cells were treated with 2, 5 and 8 μM Dioscin for 18 h. 10^6 cells of each sample were collected and washed in PBS. Then, cells were fixed in 0.4% paraformaldehyde in PBS for 5 min. After permeabilization with saponin solution (0.1% saponin in PBS containing 0.1% bovine serum albumin (BSA)), cells were labeled for 30 min at 4 °C with mouse anti-human Bcl-2 monoclonal antibody. Flow cytometry analysis was performed by FACScan (Coulter, EPICS ELITE, U.S.A.).

**RESULTS**

**Antiproliferative Effect of Dioscin on Proliferation of Hela Cells** The inhibitory effect of Dioscin on the proliferation of Hela cells was observed to occur in a dose-dependent manner (Fig. 1). At the lowest concentration (1 μM) Dioscin slightly accelerated cell growth. However the inhibition rate reached 86.8% when treated with Dioscin of 8 μM. The Hela cells proved to be sensitive to Dioscin in the dose range of 1—8 μM with an IC<sub>50</sub> value of 4.4 μM.

**Apoptosis of Hela Cells Induced by Dioscin** The percentage of apoptotic cells in the drug-treated culture was normalized relative to the untreated drug culture. Dioscin induced apoptosis as stained by Annexin V in a dose-dependent manner (Fig. 2). Approximately 70% of the cells were apoptotic when treated with 2 μM Dioscin. Among them two-
thirds were in an early stage of apoptosis and were labeled by Annexin V only. This is in contrast to the highest concentration of 8 μM at which about 90% of the apoptotic cells were in the late stage so they were stained by both Annexin V and PI.

An accumulated cell population in the sub-G1 region was observed in the Hela cells. 36.4% of the Hela cells were detected in the sub-G1 region after exposure to 5 μM Dioscin for 12 h (Fig. 3A). The obvious sub-G1 peak suggested that the cell accumulation in this region were primarily due to apoptosis. The time-course of apoptosis is shown in Fig. 3B.

**Detection of Caspase Activity** Cell lysates were evaluated for the protease activity of caspase-8, caspase-9 and caspase-3. Data were indicated as a percentage of the untreated control (Fig. 4). Caspase-9 and caspase-3 activity increased after treated with Dioscin. Compared with the greater than 6 fold increase of caspase-9, the activity of caspase-8 did not change much, suggesting that Dioscin induced apoptosis of Hela cells through the mitochondrial pathway.

**Downregulation of Bcl-2 by Dioscin** A dotted-line histogram of Bcl-2 antibody was overlaid with a histogram of isotype antibody control (Fig. 5). The fluorescent intensity of FITC conjugated on Bcl-2 antibody indicated the intracellular level of Bcl-2. The expression of Bcl-2 in Hela cells was reduced in a dose-dependent manner after treated with Dioscin.

**DISCUSSION**

Dioscin is a saponin extracted from the root of *Polygonatum Zanthlaniscianense* Pamp. Earlier results showed that HL-60 cells underwent differentiation and apoptosis after Dioscin...
treatment.\textsuperscript{10} This study detected the apoptotic effect of Dioscin on Hela cells and studied the pathway involved in apoptosis.

When treated with increasing concentrations of Dioscin, the proliferation of Hela cells was inhibited in a dose-dependent manner. Flow cytometric analysis of phosphatidylserine (PS) exposure by Annexin V staining quantified the apoptotic cells.\textsuperscript{12} Concomitant staining with PI identified cells with loss of membrane integrity that is characteristic of late apoptosis (also known as secondary necrosis). In the dosage range of 2—8 $\mu$m Dioscin the proportion of late apoptotic cells increased together with the number of apoptotic cells as a whole. At concentrations below 5 $\mu$m a small percentage of cells showed late apoptotic character, demonstrating that Dioscin had great potential to induce apoptosis in Hela cells.

Another evidence of apoptosis was provided by FACS analysis of cells in the sub-G1 region after being treated with Dioscin.\textsuperscript{13} The sub-G1 peak was obvious. Also, the viability of Hela cells after Dioscin treatment for 12 h was 100%, as judged by the trypan blue exclusion method. This result suggested that the cells in the sub-G1 region were apoptotic. A time-dependent induction of apoptosis of Hela cells was observed.

Caspases are central executioners of apoptosis in many mammalian cells. In this study, caspase-3, caspase-8 and caspase-9 were used because they are landmarks for the two major apoptotic pathways in mammalian cells.\textsuperscript{9,14,15} The low activity of caspase-8 in Hela cells treated with Dioscin demonstrated that the death-receptor pathway was not activated. This was in contrast to the high activity of caspase-9, suggesting that it was the mitochondrial pathway that contributed to apoptosis of Hela cells.

Another protein that plays an important role in the mitochondrial pathway is Bcl-2, an anti-apoptotic member of the Bcl-2 family. It key function is to regulate the release cytochrome c from mitochondria into the cytosol. Here a significant downregulation of Bcl-2 expression in Hela cells treated with Dioscin was detected. The data also confirms above results.

In conclusion, the results suggest that Dioscin is a potent apoptosis inducer in Hela cells. The induction of apoptosis results in the activation of the mitochondrial pathway. Since Dioscin has been reported to induce differentiation and apoptosis in the human leukemic HL60 cell line, Dioscin may be a potent drug for cancer treatment in clinic.

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