Gypenosides Induced G0/G1 Arrest *via* Inhibition of Cyclin E and Induction of Apoptosis *via* Activation of Caspases-3 and -9 in Human Lung Cancer A-549 Cells

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Abstract. Gynostemma pentaphyllum Makino is known in Asia for its effect on the treatment of hepatitis and cardiovascular diseases. Gypenosides (Gyp) are the major components extracted from Gynostemma pentaphyllum Makino. However, the molecular mechanism underlying the Gyp-induced cell cycle arrest and apoptotic process is unclear. In this study, the chemopreventive role of Gyp in human lung cancer (A549) cells in vitro was evaluated by studying the regulation of the cell cycle and apoptosis. Gyp induced G0/G1 arrest and apoptosis in the human lung cancer A549 cells. Investigation of the cyclin-dependent protein kinase inhibitors by Western blotting showed that p16, p21, p27 and p53 proteins were increased with the increasing time of incubation with Gyp in the A549 cells. This increase may be the major factor by which Gyp caused G0/G1 arrest in the examined cells. Flow cytometric assay and gel electrophoresis of DNA fragmentation also confirmed that Gyp induced apoptosis in the A549 cells. Our data demonstrated that Gyp-induced apoptotic cell death was accompanied by up-regulation of Bax, caspase-3 and

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caspase-9, but down-regulation of the Bcl-2 levels. Taken together, Gyp appears to exert its anticancer properties by inducing G0/G1-phase arrest and apoptosis via activation of caspase-3 in human lung A549 cancer cells.

Many experimental studies have demonstrated that dietary agents (common fruits and vegetables) exhibit anticancer activities, but their function and active components are still unclear (1, 2). Gypenosides (Gyp) are extracted from *Gynostemma pentaphyllum* Makino, a popular folk medicine that has long been used in Chinese populations for treating diseases, such as hepatitis (3), hyperlipoproteinemia (4, 5), cardiovascular disease (6) and cancer (7). Numerous experiments have indicated that Gyp possesses anti-inflammatory (8), antithrombotic (9), antioxidative (10) and anticancer (11-14) activity.

In our laboratory, it has been demonstrated that Gyp induced apoptosis through the activation of caspase-3, promoted the levels of Bax and decreased the levels of Bcl-2 in liver cancer cells (14), and it has also been found that Gyp inhibited N-acetyltransferase gene expression and protein levels in human cervical cancer cells (15). Recently, we have found that Gyp induced G0/G1 arrest and apoptosis through the mitochondrial-dependent pathway in human colon cancer cells (16). One of the most effective strategies for anticancer (cancer cell killing) agents is the induction of cell cycle arrest and apoptosis. The effect of Gyp on human lung cancer A549 cells remains unclear. Therefore, the mechanisms underlying the induction of cell cycle arrest and apoptosis were investigated in human lung cancer A549 cells.

Materials and Methods

Chemicals and reagents. Propidium iodide (PI), ribonuclease-A, sodium bicarbonate, Triton X-100, Tris-HCl and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). DMEM medium with 2 mM L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human lung cancer cell line (A549). The human lung carcinoma cell line, A549, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were immediately placed into 75-cm³ tissue culture flasks with F-12K medium with 2 mM L-glutamine supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% glutamine and grown at 37°C in a humidified 5% CO₂ and 95% air atmosphere (16, 17).

The effect of Gyp on cell morphology and viability. The A549 cells were placed in 12-well plates at a density of $5x10^5$ cells/well and incubated at 37°C for 24 h. Various concentrations of Gyp (0, 100, 150, 200, 300 and 400 µg/mL) were added and the cells were incubated for various periods of time. For cell morphology, the cells in the plate were examined by using a phase-contrast microscope and were photographed (16, 17). To determine cell viability, the flow cytometric protocol was used, as previously described (16, 17).

The effects of Gyp on the cell cycle and sub-G1 group. Approximately $5x10^5$ A549 cells/well/12-well plate were incubated with various concentrations of Gyp or 300 µg/mL Gyp for various time-periods The cells were then harvested by centrifuging and the percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined using flow cytometry, as described previously (16, 17).

Gyp-induced DNA fragmentation (apoptosis). Approximately $5x10^{6}$ A549 cells/ml were treated with or without Gyp at 300 µg/mL for different periods of time (12 or 24 h), before, extracting and analysing DNA by gel electrophoresis, as described previously (18).

Examination of proteins associated with the cell cycle and apoptosis. Approximately $5x10^6$ A549 cells/ml were treated with Gyp at 300 µg/mL (control, without Gyp) for 6, 12, 24 or 48 h, then harvested, lysed and the proteins associated with the cell cycle were detected by Western blot. All the samples were analyzed by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (16-18).

Reverse transcriptase polymerase chain reaction (RT-PCR) examination of caspase-3 and -9, Bcl-2 and Bax gene expression in A549 cells. The total RNA was extracted from the A549 cells after exposure to 300 µg/mL Gyp for 6, 12, 24 and 48 h, by using a Qiagen RNeasy Mini Kit. The entire protocol for RT-PCR has been described previously (19-20). The sequence of primers was as follows: forward: TATGGTTTTGTGATGTTTGTCC, reverse: AGATCCAGGGG CATTGTAG for caspase-3 (GenBank accession, G10724); forward: ATAACCTTTTAGGCTGGTGG, reverse: AGAGCAGAAAGA GGTGAGAGA for caspase-9 (GenBank accession, G17703); forward: CTGATAACTTGG GGAGGCAA, reverse: GAGAGTCC AACTGCAAAGGC for Bcl-2 (GenBank accession, G06794) and forward: GGGGAT TTCTGACTTGAGG, reverse: TTGGGCTGA TTTGATTTCTG for Bax (GenBank accession, D29582).

Statistical analysis. Student's *t*-test was used for the statistical analysis between the Gyp-treated and control groups. A *p*-value < 0.01 was considered significant.

Results

Effect of Gyp on cell morphology and viability. Dose- and time-dependent effects of Gyp are shown in Figure 1A and B. The cells were increasingly morphologically-changed by increasing Gyp concentration, while there was a lower percentage of viable cells as the time of incubation and concentration of Gyp increased.

Effect of Gyp on cell cycle arrest and apoptosis. The flow cytometric analysis indicated that Gyp increased the percentage of cells in G0/G1 (Figure 2A and B). The sub-G1 groups also appeared in the cell cycle, which meant that Gyp induced apoptosis in these cells (Figure 2C). Increased time of Gyp treatment led to an increase in G0/G1- and sub-G1-phases in the A549 cells.

Gyp-induced DNA fragmentation (apoptosis). The results from DNA gel electrophoresis indicated that Gyp induced DNA fragmentation (Figure 3).

Effect of Gyp on the expression of cell cycle- and apoptosisassociated proteins. Figure 4 A and B1 show the cell cycleassociated proteins (cyclin A, B, D1/3 and E; cyclin-dependent protein kinase (CDK1), CDK2 and CDK4, cyclin-dependent protein kinase inhibitor (CDK1) (p16, p21, p27 and p53), and Figure 4 C, D, E and F show the apoptosis-associated proteins (caspase-3 and -9, Bcl-2 and Bax) by Western blotting and PCR. The levels of cyclins A and E, CDK2 and -4 were decreased, while the levels of cyclin B, cyclin D1/3, CDK1 and -4 were increased and the levels of G0/G1 associated proteins, such as p16, p21, p27 and p53, were increased. Furthermore, Gyp increased the protein levels and gene expressions of caspase-9, caspase-3 (panel C and D) and Bax (panel E and F); these effects may have led to apoptosis.

Discussion

Previous studies have demonstrated that Gyp affected Nacetyltransferase (NAT) activity and gene expression in human cervical cancer cells (15) and induced cell cycle arrest and apoptosis in human colon cancer colo 205 cells (16).

Phase-contrast microscopy indicated that Gyp induced morphological changes in the A549 cells and the flow

(A)





Figure 1. Effect of Gyp on cell morphology and viability of human lung cancer A549 cells. Cells ($5x10^5$ cells/well; 12-well plates) were cultured in DMEM + 10% FBS. (A) Phase-contrast microscopy after 24 h incubation with various concentrations of Gyp and (B) percentage of viable cells determined by flow cytometry, after incubation with various concentrations of Gyp for various times. Each point is mean \pm S.D. of three experiments; *p < 0.01.

cytometric assays indicated that treatment with Gyp induced G0/G1-phase arrest and apoptosis. The analysis of DNA content versus light scatter of the Gyp-treated cells indicated that apoptosis followed Gyp induced G0/G1-phase arrest (Figure 2B). Cells normally progress from the G1- to the S-phase, which is regulated by CDK2 associated with cyclin E (21, 22) and CDK inhibitors. When p53 was promoted it may

have led to activation of protein kinase c-mediated p53 gene transcription followed by the G1-phase arrest and promoted cellular repair mechanisms in mock cells (23, 24). In the present study Gyp increased p53, p16, p21 and p27 protein levels which may have caused the G0/G1-phase arrest in the A549 cells. The p27 protein is known to be a CDK inhibitor which negatively regulates cyclin-CDK complexes at the G1-S



Figure 2. Effect of Gyp on cell cycle arrest and apoptosis in human lung cancer A549 cells. Cells ($5x10^5$ cells/well; 12-well plates) were exposed to various concentrations of Gyp for 48 or 60 h 300 µg/mL Gyp for 0, 12, 24, 48 and 72 h the cells were harvested and cell cycle (A: representative profiles; B: percent of cells per phase) and sub-G1 group (C: percent of cells in apoptosis) by flow cytometry. Data represents mean±S.D. of three experiments; *p<0.01.

transition leading to inhibition of entry into the S-phase from G0/G1 of the cell cycle. It is known that cancer cells show low p27 expression which is correlated with tumor grade, recurrence rate and prognosis in several carcinomas (25, 26). The DNA gel electrophoresis confirmed that Gyp induced apoptosis in the A549 cells (Figure 3) based on the occurrence



Figure 4. Representative Western blots and PCR of cell cycle and apoptosis-associated proteins in A549 cells after exposure to Gyp. The A549 cells (5x10⁶ cells/well) were treated with Gyp at 300 µg/mL for 6, 12, 24 or 48 h. The levels of cyclins A, B, D1/3 and E, CDK1, CDK2 and CDK4 (panel A), p16, p21, p27 and p53 (panel B), caspase-3 and -9 (panel C), Bcl-2 and Bax (panel E) expressions were estimated by Western blotting. For PCR examinations, A-549 cells were treated with Gyp at 300 µg/mL for 6, 12, 24 and 48 h, total RNA extracted and subjected to PCR. Caspase-3 and -9 (panel D), Bcl-2 and Bax (panel F) (CT: control; GP: Gypenosides).

of DNA fragmentation which was in agreement with our previous (16) and other reports (27).

Our results also showed that Gyp increased the protein levels and gene expression of Bax and decreased the protein levels and gene expression of Bcl-2 which may have led to apoptosis. It is well-known that the Bcl-2 family plays an important role as an intracellular suppressor of apoptosis with a cyto-protective function in cells (28) and high expression levels of ectopic Bcl-2 protein have been related to resistance to apoptosis *via* the prevention of glutathione



Figure 5. Proposed signal pathways on Gyp-induced apoptosis in human lung cancer A549 cells.

(GSH) depletion, cytochrome c release, and mitochondrial dysfunction (29-31). Bax also plays an important role in the promotion of apoptosis (32) and it is well-known that the ratio of Bcl-2 to Bax is associated with the sensitivity or resistance of a cell to the process of apoptosis (33). It has also been shown that the accumulation of cells in G0/G1-phase arrest 24-120 h after transduction of the p53 or p27/kip genes was associated with an increase in early apoptosis (34-36).

The results from Western blotting and PCR demonstrated that Gyp promoted the levels of protein and gene expression of caspase-3 and -9. Sequential activation of caspase-9 and -3, but not the other caspases, was involved in Gyp-induced apoptosis. The inhibitor of caspase-3, z-VAD-fmk was also used and blocked apoptosis in the A549 cells (data not shown). Caspase-9 inhibition by the specific inhibitor, Ac-LEHD-fmk, caused a decrease in caspase-3 enzyme activity during Gyp treatment (data not shown). This was in agreement with our earlier experiments which demonstrated that Gyp induced apoptosis in human colon

cancer colo 205 cells through caspase-3 activation (16). Apoptosis induced by Gyp was associated with the release of cytochrome c to the cytosol, caspase activation, a decrease in Bcl-2 protein and an increase in Bax protein levels. The possible pathways of Gyp-induced apoptosis in human lung cancer A549 cells are illustrated in Figure 5.

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