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Coumarin Induces Cell Cycle Arrest and Apoptosis in Human Cervical Cancer HeLa Cells through a Mitochondria- and Caspase-3 Dependent Mechanism and NF-κB Down-regulation

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Abstract. *The effects of coumarin on cell viability, cell cycle arrest and induction of apoptosis were investigated in human cervical cancer HeLa cells. Coumarin was cytotoxic with an IC_{50} of 54.2 μM, induced morphological changes, and caused G0/G1 arrest and apoptosis. The decreasing number of viable cells appeared to be due to induction of cell cycle arrest and apoptotic cell death, since coumarin induced morphologically apoptotic changes and internucleosomal DNA laddering fragmentation and increased the sub-G1 group. Coumarin affected the production of reactive oxygen species and Ca^{2+} concentration, and dose-dependently induced the depolarization of mitochondrial membrane potential. Also, coumarin treatment gradually decreased the expression of G0/G1-associated proteins which may have led to the G0/G1 arrest, and the anti-apoptotic proteins Bcl-2 and Bcl-xL, and increased the expression of the pro-apoptotic protein Bax. Coumarin decreased the mitochondrial membrane potential and promoted the release of cytochrome c and the activation of caspase-3 before leading to apoptosis. These results provide information on the mechanisms by which coumarin induces cell cycle arrest and apoptosis in human cervical cancer cells (HeLa).*

It has been reported that some coumarin compounds, including coumarin and 7-hydroxycoumarin, inhibit the cell growth of various types of cancer cell lines (1-3). Many investigators have also reported on the use of coumarin (1,2-benzopyrone), or its metabolite 7-hydroxycoumarin, for the treatment of some human carcinomas (4-7). No adverse effects of coumarin have been reported in humans using doses up to 7 g daily, after two weeks of continued treatment (8, 9). In the present study, the effect of coumarin on cell cycle arrest and induction of apoptosis in human cervical cancer HeLa cells, for which no information is currently available, was examined.

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Materials and Methods

Chemicals and reagents. Coumarin, Tris-HCl, 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA), dihexyloxacarbocyanine iodide ($DiOC_6$) and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO) and TE buffer were purchased from Merck Co. (Darmstadt, Germany). MEM with Earle's BSS medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human cervical epithelioid carcinoma cell line (HeLa). The human cervical epithelioid carcinoma cell line, HeLa, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC) and the cells were immediately placed into 75-cm^3 tissue culture flasks with MEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% glutamine, and grown at 37˚C in a humidified 5% CO_2 and 95% air atmosphere.

Cell morphology and viability. The HeLa cells were placed in 12-well plates at a density of $5x10^3$ cells/well and incubated at 37˚C for 24 h. The various concentrations of coumarin (0, 10, 20, 50, 75 and 100 μM) were added and the cells were incubated for various periods of time. DMSO (solvent) was used for the control regimen. For cell morphology, cells were examined under a phase-contrast microscope and photographed (10). To determine cell viability, the flow cytometric protocol was used, as previously described (10-13).

Flow cytometry analysis of cell cycle and apoptosis. Approximately $5x10^5$ HeLa cells/well in 12-well plates were incubated with various

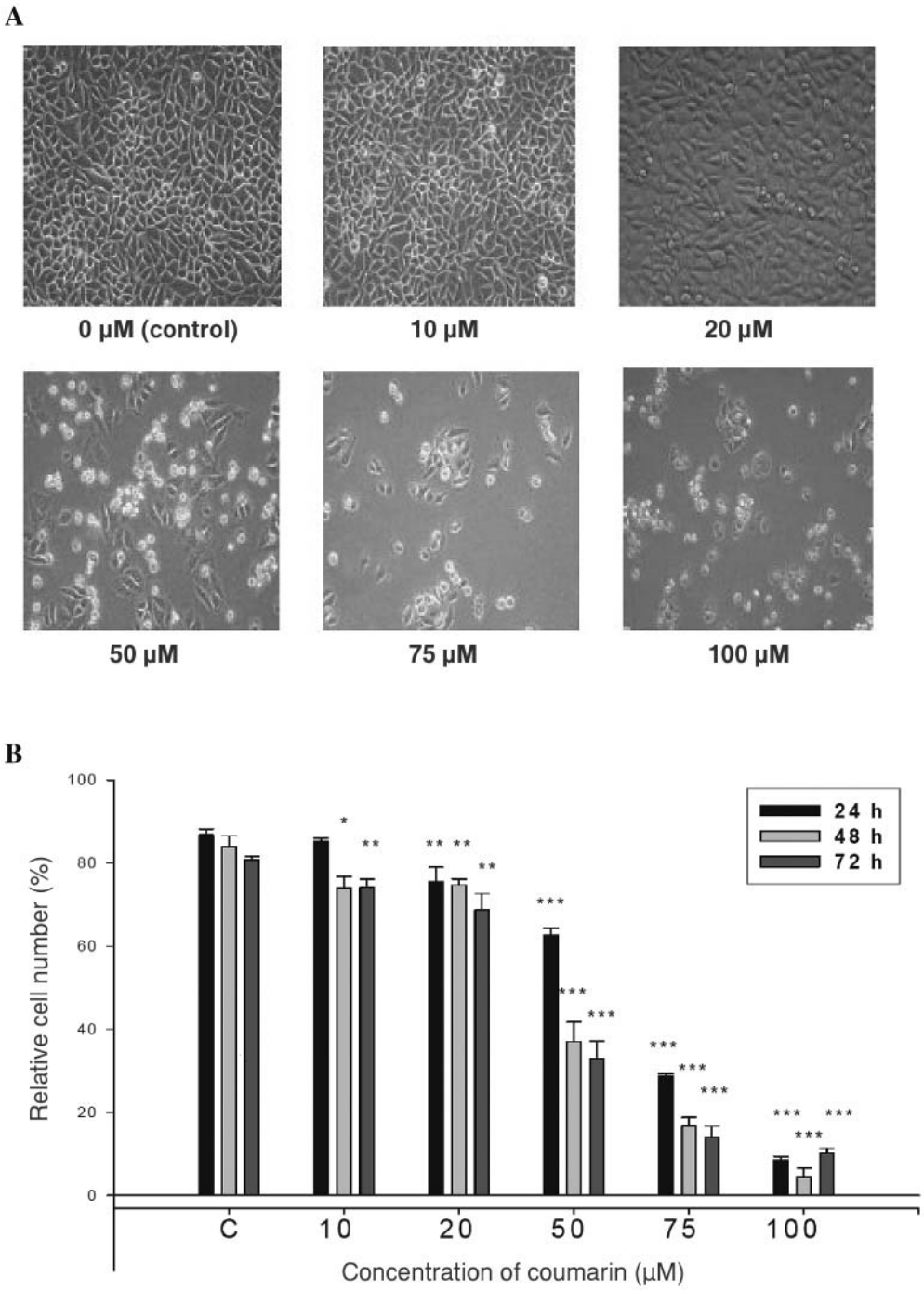

Figure 1. *Morphological changes and percentage of the viable HeLa cells after coumarin treatment. The cells were photographed (A) and collected by centrifugation and the number of viable cells (B) was determined by trypan blue exclusion and flow cytometry. Each point is mean±S.D. of three experiments. *Significant differences between coumarin-treated cells and control *p<0.05.*

concentrations (0, 1, 5, 10, 50 and 100 μM) of coumarin for 0, 12, 24, 48 and 72 h before the cells were harvested by centrifuging and the percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined by flow cytometry, as described previously (11-13).

DNA gel electrophoresis examination of coumarin-induced DNA fragmentation (apoptosis). Approximately 5x10^6 HeLa cells/ml were treated with coumarin at 100 μM for different periods of time (0, 24, 48 and 72 h) before isolating the cells to extract the DNA which was subjected to gel electrophoresis, as described previously (11-13).

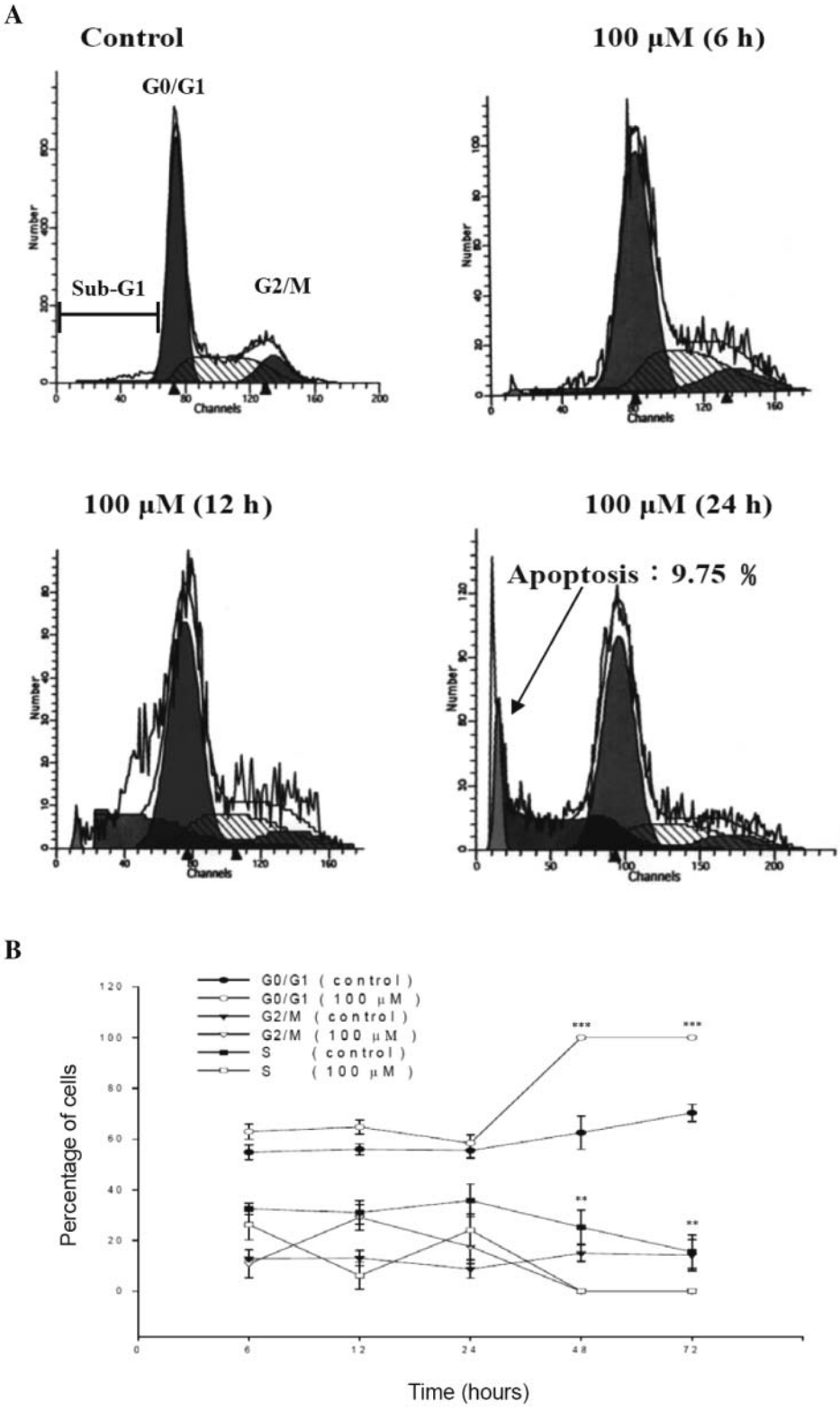

Figure 2. *Flow cytometric analysis of the coumarin effects on HeLa cell cycle and sub-G1 group. A: representative profiles of cell cycle. B: percentage of cells in various phases. Data represents mean±S.D. of three experiments. *Significant differences between coumarin-treated cells and control *p<0.05.*

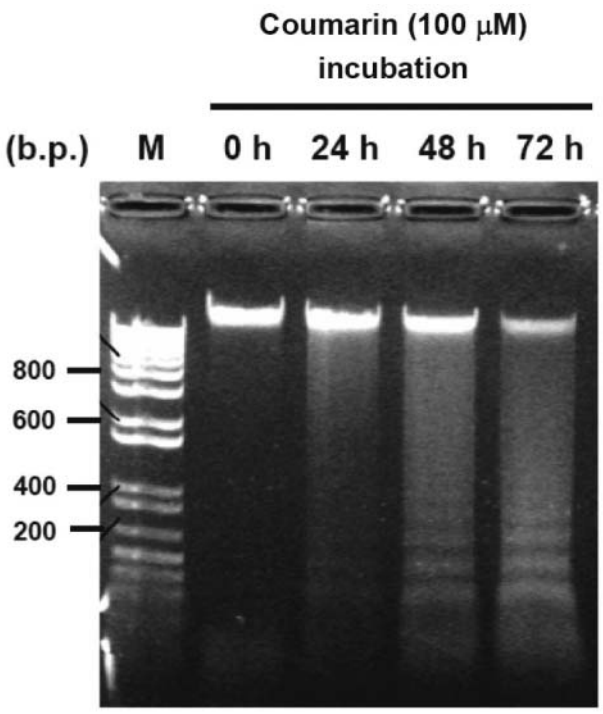

Figure 3. *Gel electrophoresis for DNA fragmentation in HeLa cells after treatment with coumarin. Cells (5x10^6 cells/well; 12-well plates) were incubated with 100 μM coumarin for different periods of time (0, 24, 48 and 72 h), then DNA was extracted, and the DNA fragmentation was examined by DNA gel electrophoresis (M: DNA Ladder Marker).*

Flow cytometry of reactive oxygen species (ROS) using 2,7-dichlorodihydro-fluorescein diacetate staining. Approximately 5x10^5 HeLa cells/ml were treated with coumarin at 0, 1, 5, 10, 25, 50 and 100 μM for 24 h incubation. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM) (Sigma) and incubated at 37˚C for 30 min to detect the changes of ROS by flow cytometry as described previously (Becton Dickinson FACS Calibur) (13, 14).

Flow cytometry of Ca^{+2} by using Indo 1/AM staining. Approximately 5x10^5 HeLa cells/ml were treated with coumarin at 0, 1, 5, 10, 25, 50 and 100 μM for 24 h before isolating the cells to detect any changes in Ca^{+2} concentration. The cells were harvested by centrifuging and washed twice before re-suspension in Indo 1/AM (3 μg/ml) (Calbiochem, La Jolla, CA, USA) incubated at 37˚C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (13, 15).

Flow cytometry of mitochondrial membrane potential using $DiOC_6$ staining. Approximately 5x10^5 HeLa cells/ml were treated with coumarin at 0, 1, 5, 10, 25, 50 and 100 μM for 24 h to detect changes in the mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L), incubated at 37˚C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (13, 16).

Western blotting of proteins associated with the cell cycle and apoptosis. Approximately 5x10^6 HeLa cells/ml were treated with coumarin at 0, 10, 50 and 100 μM for 24 or 48 h, before isolating and lysing the cells and then quantifying the proteins associated with the cell cycle and apoptosis. All samples were separated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis, as described previously (10-14). The primary antibodies were cyclin D1, Cdk2, p15, Cdc25A, p21, p53, Bcl-xL-xS, Bcl-2, Bax, cytochrome c, caspase-3, -9, NF-κB p50, NF-κB p65, iNOS and β-actin.

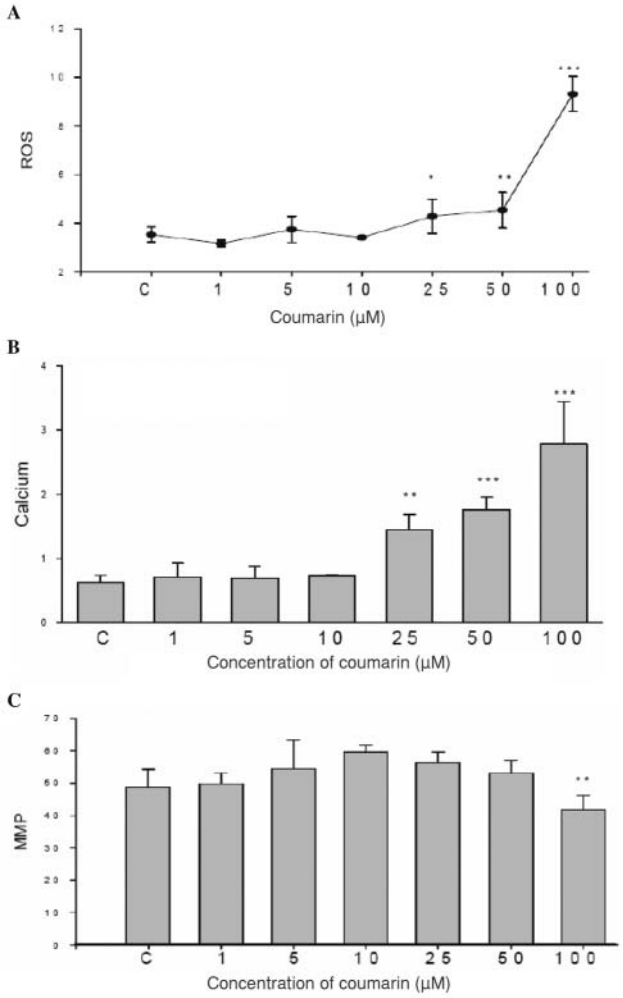

Figure 4. *Flow cytometric analysis of reactive oxygen species (ROS), Ca^{2+} concentration and mitochondrial membrane potential ($\Delta\Psi m$) in HeLa cells treated with coumarin. Percentage of stained cells determined by flow cytometry for A: ROS (DCFH-DA staining), B: Ca^{2+} (Indo-1/Am staining) and C: $\Delta\Psi m$ ($DiOC_6$ staining) for various concentrations of coumarin (% of cells being stained). Significant differences between coumarin-treated cells and control *p<0.05, **p<0.01, ***p<0.001.*

Results

Cell morphology and viability. Coumarin caused morphological changes in the HeLa cells and there were fewer

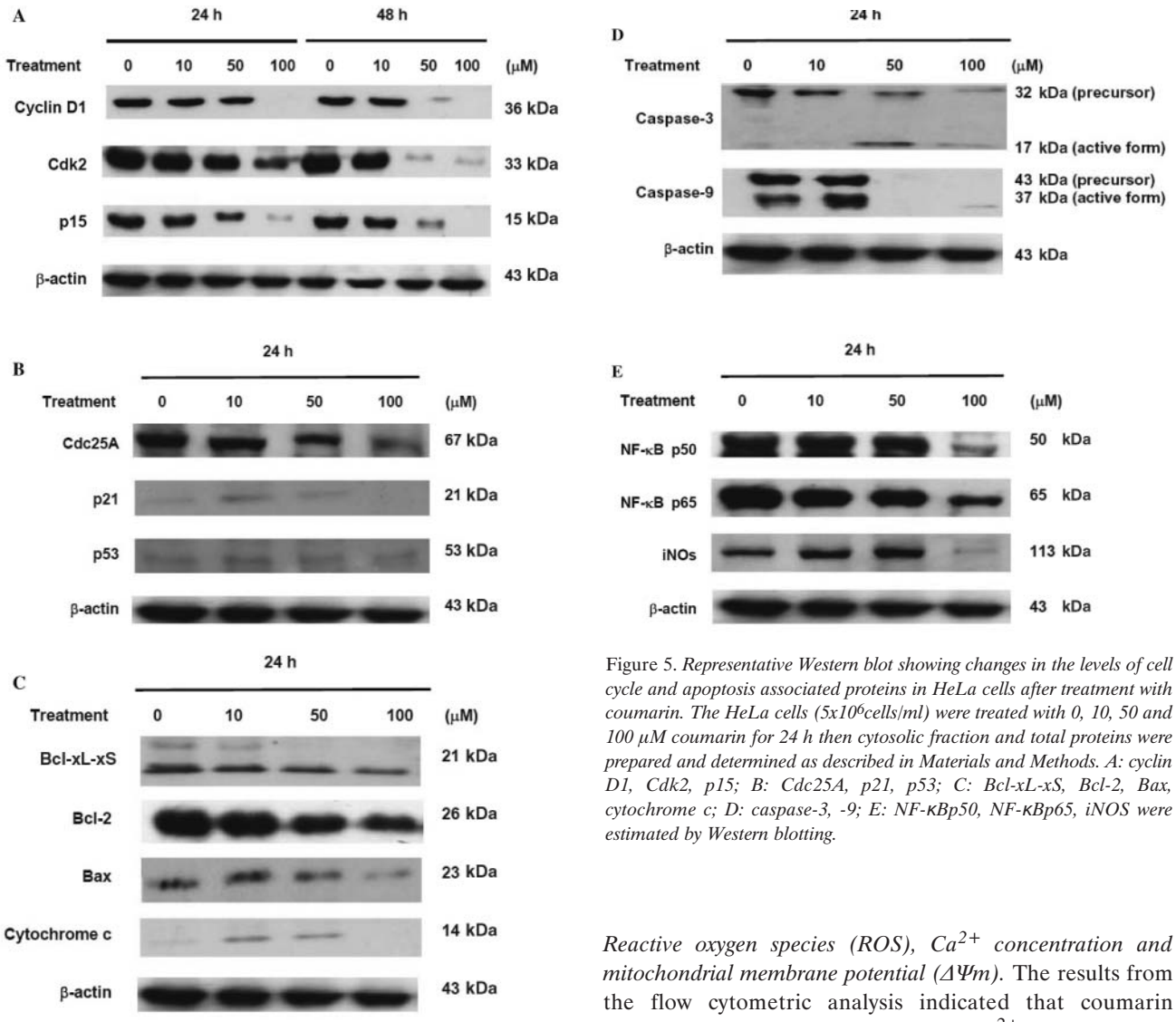

Figure 5. *Representative Western blot showing changes in the levels of cell cycle and apoptosis associated proteins in HeLa cells after treatment with coumarin. The HeLa cells (5x10^{6}cells/ml) were treated with 0, 10, 50 and 100 μM coumarin for 24 h then cytosolic fraction and total proteins were prepared and determined as described in Materials and Methods. A: cyclin D1, Cdk2, p15; B: Cdc25A, p21, p53; C: Bcl-xL-xS, Bcl-2, Bax, cytochrome c; D: caspase-3, -9; E: NF-κBp50, NF-κBp65, iNOS were estimated by Western blotting.*

viable cells as coumarin concentration increased. Coumarin was cytotoxic to the HeLa cells with an IC_{50} of 54.2 μM. (Figure 1A and B).

Cell cycle arrest and apoptosis. The results from flow cytometric analysis indicated that after 24 h coumarin treatment, there was an increase in the percentage of cells in G0/G1 (enhanced G0/G1 peak) and the sub-G1 groups also appeared in the cell cycle, which meant that coumarin induced apoptosis in HeLa cells (Figure 2A and B).

DNA damage. DNA gel electrophoresis indicated that coumarin-induced DNA laddering fragmentation, and apoptosis effects were time-dependent (Figure 3).

Reactive oxygen species (ROS), Ca^{2+} concentration and mitochondrial membrane potential ($\Delta\Psi m$). The results from the flow cytometric analysis indicated that coumarin promoted the production of ROS and Ca^{2+} (Figure 4A and B) but decreased the levels of $\Delta\Psi m$ (Figure 4C) in HeLa cells, at a dose-dependent manner.

G0/G1 and apoptosis associated proteins associated proteins. The results indicated that coumarin decreased the protein levels of cyclin D1, Cdk2, p15 and Cdc25A, but increased p21 and p53 proteins (Figure 5A and B). Coumarin decreased the levels of Bcl-xL-xS and Bcl-2, but increased the levels of Bax, cytochrome *c*, and the active forms of caspase-3 and caspase-9 (Figure 5C and D). However coumarin also decreased the levels of NF-κB p50 and NF-κB p65 , but increased iNOS protein (Figure 5E).

Discussion

Many experiments have demonstrated that the cytostatic effect of the coumarin compounds in non-small lung cancer

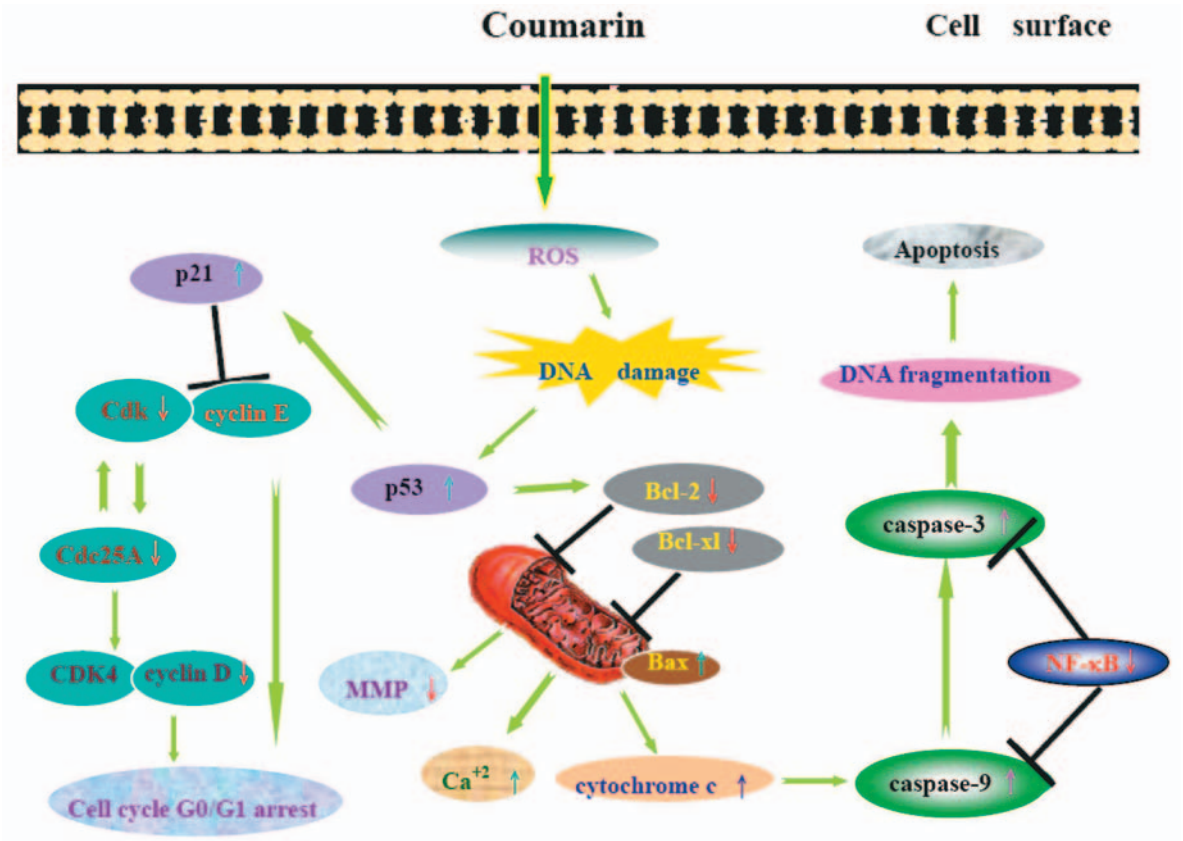

Figure 6. *Proposed model of mechanisms for coumarin-induced cell cycle arrest and apoptosis in HeLa cells. Coumarin decreased cyclin D1, Cdk2, Cdc25A expression which led to G0/G1 arrest. Coumarin increased the production of Bax, caspase-3 and -9, and decreased Bcl-2 which caused apoptosis in HeLa cells.*

cell lines are associated with cell growth inhibition observed both *in vitro* and *in vivo* (1, 3, 5, 17). It has also been reported that coumarin and 7-hydroxycoumarin reduce the expressions of Ras and Myc which are associated with cell proliferation in ras- or myc-transfected murine fibroblasts (18, 19). In the present study coumarin induced morphological changes and decreased the percentage of viable HeLa cells. It also induced G0/G1 arrest in a dose-dependent manner. These data are in agreement with the report of Kahn *et al.* (19) which demonstrated that coumarin caused G1-phase arrest in the MTV-EJ ras cell line. In order to define the signaling pathway that led to the cell cycle arrest in the G1-phase, we also used Western blotting to show that coumarin inhibited the protein levels of cyclin D1, Cdk2 and Cdc25A (Figure 5A and B).

Our results from flow cytometric analysis (Figure 2A) and DNA gel electrophoresis (Figure 4) indicated that coumarin induced apoptosis, based on the sub-G1 group demonstrated in the cell cycle analysis and the DNA laddering fragmentation shown by DNA gel electrophoresis. These results are in agreement with other reports which have demonstrated that coumarin induced apoptosis in human non-small cell lung carcinoma (NSCLC) cell lines (20, 21). Our data are also the first to show that coumarin increased the Ca^{2+} concentration and ROS production, and decreased mitochondrial membrane potential ($\Delta\Psi m$) (Figure 3A, B and C). Those factors appear to be associated with coumarin-induced apoptosis. ROS production may cause DNA damage and affect the Ca^{2+} levels in the Endoplasmic reticulum (ER) leading to the occurrence of ER stress. Coumarin also decreased the $\Delta\Psi m$ which might have led to cytochrome c release which then caused the activation of caspase-3 followed by apoptosis. Western blotting also demonstrated that coumarin decreased the protein levels of Bcl-xL and Bcl-2 which are anti-apoptotic factors. Coumarin also decreased the levels of NF-κB p50 and p65, and increased the levels of iNOS (Figure 5E). Low dose (10 μM) coumarin slightly increased, the protein level of p53 (Figure 5D) indicating that the apoptosis induced by coumarin in the HeLa cells was through the p53-dependent pathway. The proposed model for coumarin induced apoptosis is summarized in Figure 6. Coumarin induces G0/G1 arrest

and apoptosis *via* a mitochondria-dependent pathway and affects NF-κB protein levels in HeLa cells. Further investigations *in vivo* are needed before coumarin can become a new option in the treatment of cervical cancer.

Acknowledgements

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