Abstract. The present studies were undertaken to analyze the factors regulating 3-hydroxycinnamic acid-induced apoptosis and cell cycle arrest. Treatment of human cervix HeLa cells with 3-hydroxycinnamic acid induced apoptosis and G0/G1-phase arrest. The percentage of apoptosis induced by 3-hydroxycinnamic acid in HeLa cells was increased with incubation time. The results also demonstrated that 3-hydroxycinnamic acid increased the expression of p53, caspase-3, Bax and cyclin B. These results demonstrated that 3-hydroxycinnamic acid induced apoptosis through p53- and caspase-3-dependent pathways.

It is now well documented that induction of cell cycle arrest and apoptosis by anticancer agents is an important strategy for killing cancer cells. Anticancer drugs usually induce cytotoxicity via activation of signal pathways which cause programmed cell death (apoptosis). A series of studies showed that clinical anticancer agents such as paclitaxel (Taxol) acted through G2/M arrest and the induction of apoptosis in human cancers (breast, ovary, lung, head and neck) (1, 2).

Cervical cancer is a major cause of morbidity in women worldwide. Cervical cancer has long been considered a poorly chemosensitive tumor. The role of chemotherapy in the treatment of cervical cancer has mainly been confined to persistent or recurrent disease after failure of surgery and/or radiotherapy. Through the management of cervical cancer, chemotherapy has received increasing attention in the last two decades. Cisplatin represents the cornerstone of chemotherapy for cervical cancer, although chemotherapy for this cancer still seems unsatisfactory (3, 4).

3-Hydroxycinnamic acid is one of the substituents of 3,4-dihydroxycinnamic acid (caffeic acid, CAF), which is a natural product containing a catechol group with an a,b-unsaturated carboxylic acid chain, that has shown hepatoprotective properties (5). It was reported that 3-hydroxycinnamic acid reduces the acute liver damage markers produced by CCl4 administration (6). However, there is no available information to address the effect of 3-hydroxycinnamic acid on human cancer cells in vitro. Therefore, in the present paper, we investigated the effects of 3-hydroxycinnamin acid on the basal expression of some genes related to G0/G1 arrest and apoptosis in human cervix epithelial carcinoma HeLa cells and also the variation in gene expression induced by exposure of the cells to 3-hydroxycinnamic acid treatment.

Materials and Methods

Chemicals and reagents. 3-Hydroxycinnamic acid, propidium iodide (PI), Tris-HCl, triton X-100, ribonuclease-A and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphate and TE buffer were purchased from Merck Co. (Darmstadt, Germany). Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human cervix cancer cell line. The human cervix epithelial carcinoma cell line (HeLa: cervix, adenocarcinoma, 31 years, female) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm2 tissue culture flasks and grown at 37°C in a humidified 5% CO2 and 95% air atmosphere, in MEM medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine.

Cell morphological changes were examined by contrast-phase microscope and cell viability was determined by using flow cytometry. HeLa cells were plated in 12-well plates at a density of 5x104 cells/well and grown for 24 h. 3-Hydroxycinnamic acid (0.1, 1, 2, 5...
Cell proliferation was determined by MTT assays. The cells (HeLa) were subcultured into a 96-well plate with 1x10^4 cells per well in medium, at 37 °C, 5% CO₂ and 95% air atmosphere, before being treated with or without various concentrations of 3-hydroxycinnamic acid (0.1, 0.5, 1, 2, 3 and 5 mM), each in triplicate for 24 h. At the end of the incubation, the cells were harvested and washed with PBS. Twenty μl of MTT was added to each well and incubated for 2 h before 200 μl DMSO was added. The absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 570 nm, with a reference wavelength of 630 nm (8).

Flow cytometry analysis of DNA content for cell cycle and apoptosis in HeLa cells treated with 3-hydroxycinnamic acid. HeLa cells (2x10^5 cells/well) in 12-well plates were incubated with 5 or 10 mM 3-hydroxycinnamic acid for different time-periods before the cells were harvested. The cells were fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase A (Sigma) and 0.1% triton x-100. After 30 min at 37 °C in the dark, the cells were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Then cell cycle and apoptosis were determined and analyzed as previously (9).

DNA fragmentation electrophoresis analysis. HeLa cells were plated in 10-cm dishes at a density of 5x10^6 cells/well and grown for 24 h. They were then treated with 5, 10 and 25 mM 3-hydroxycinnamic acid while only adding DMSO (solvent) for the control regimen and grown at 37 °C, in a humidified 5% CO₂ for 72 h for DNA fragmentation electrophoresis assay. The DNA was prepared using the genomic DNA isolation kit protocol (BIO 101, La Jolla, CA, USA). The loading buffer [10 mM EDTA, 0.2% (w/v) bromophenol blue and 50% (v/v) glycerol] was individually added to each DNA sample set at about a ratio of 1.5. Approximately 50 μl of DNA was loaded into each well and 1.0% agarose electrophoresis was carried out at 50 V in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002M EDTA, pH 8.0) for 30 min. After electrophoresis, DNA was visualized by soaking the gel in the TBE buffer containing 0.1 mM ethidium bromide. The DNA in the gel was observed by using UV light and was photographed (7).

Western blot analysis. About 5x10^6 cells (HeLa), with or without various concentrations of 3-hydroxycinnamic acid cotreatment, were harvested and were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1μg/ml aprotinin, 1 mg/ml leupeptin) for 30 min at 4 °C, followed by centrifugation at 10,000 rpm for 30 min. Fifty μg of total protein was electrophoresed on 10% SDS-polyacrylamide gels and transferred onto Immobilon™-P transfer membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked in 1x PBS-T containing 5% (w/v) non-fat milk and 0.2% Tween-20, and then incubated with mouse anti-human cyclin B1, p53, Bax, caspase-3 and β-actin (Upstate, Lake Placid, NY, USA) at room temperature for 1 h. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech)(7).

Statistical analysis. The Student’s t-test was used to analyze the statistical significance between the 3-hydroxycinnamic acid-treated and control groups.

and 10 mM) was added and the cells grown for 24 h. DMSO (solvent) was used for the control regimen. For cell morphological changes, cells were photographed by contrast-phase microscope. For determining cell viability, the flow cytometric protocol was used, as previously described (7).
Results

Effects of various concentrations of 3-hydroxycinnamic acid on cell morphology and viability of human cervix cancer HeLa cells. In the presence of 3-hydroxycinnamic acid (0.5, 1, 2, 5 and 10 mM), HeLa cells were collected and stained by PI and analyzed by flow cytometry. The results indicated that cell survival decreased as the concentration and time increased (Figures 1A and B). These data suggested that 3-hydroxycinnamic acid induced cell death on HeLa cells. An increase in the concentration of 3-hydroxycinnamic acid resulted in increasing morphological changes (cell death), and a greater decrease of viable HeLa cells were confirmed by MTT methods (Figure 2 and Figure 1C).

3-Hydroxycinnamic acid induced cell cycle arrest and apoptosis in human cervix cancer HeLa cells. The flow cytometry results for cell cycle analysis indicated that, during the 72-h time-period, 3-hydroxycinnamic acid increased the percentage of cells in G0/G1 (enhanced G0/G1 peak) and decreased the percentage of cells in G2/M. The control cells showed a normal pattern of DNA content that reflected G0/G1, S- and G2/M-phases of the cell cycle (Figure 3A, left panel). The 3-hydroxycinnamic acid-treated cells showed a typical apoptosis pattern of DNA content that reflected G0/G1, S- and G2/M-phases of the cell cycle, together with a sub-G0/G1-phase (corresponding to apoptotic cells), as shown in Figure 3A (right panel), while the ratios of cell cycle are presented in Figure 3B. A pre-G0/G1 apoptotic peak was very clear after the cells were treated for 72 h. The percentage of apoptotic cells in various time-periods of 3-hydroxycinnamic acid treatment are shown in Figure 3C. An increase of 3-hydroxycinnamic acid concentration led to an increase of apoptosis in HeLa cells.

DNA laddering fragmentation occurred after HeLa cells were treated with 3-hydroxycinnamic acid. DNA from treated and non-treated 3-hydroxycinnamic acid cells was examined in 0.8% agarose gel electrophoresis (Figure 4). The DNA from control cells was not degraded and increased amounts of DNA laddering fragments occurred in cells treated with 3-hydroxycinnamic acid.

Effects of 3-hydroxycinnamic acid on p53, caspase-3, Bax and cyclin B proteins in HeLa cells. Changes in p53, caspase-3, Bax and cyclin B levels in response to varying concentrations of 3-hydroxycinnamic acid were examined and determined by Western blotting (Figs. 5A and B). It was demonstrated that p53, caspase-3, Bax and cyclin B levels increased after 2, 5 and 10 mM 3-hydroxycinnamic acid treatment. These effects of 3-hydroxycinnamic acid on p53, caspase-3, Bax and cyclin B were also dose-dependent. These results also demonstrated that 3-hydroxycinnamic acid induced apoptosis via the p53 and caspase-3 pathways in HeLa cells.

Discussion

The aim of this work was to determine the effects of 3-hydroxycinnamic acid on cell cycle arrest, cytotoxicity and apoptosis with associated factors on a human cervix cancer cell line (HeLa). Although the metabolism of 3-hydroxycinnamic acid is unknown, it is known that E. coli K12 grew with 3-hydroxycinnamic acid as the sole source of carbon (10). The reason for selecting human cervix cancer cells is the increasing prevalence of this cancer, with 8.55 persons per 100,000 dying annually due to cervical cancer in Taiwan. Cancer chemoprevention, as first defined by Sporn in 1976, uses natural, synthetic, or biological chemical agents to reverse, suppress, or prevent carcinogenic progression (11). It is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis. To date, the chemotherapy for this cancer is unsatisfactory. Over the past 30 years, several agents have been tested but cisplatin and ifosfamide are the agents that have attracted the greatest attention. Cisplatin represents the cornerstone of chemotherpay for cervical cancer. The common features for chemotherapeutic agents are the induction of cell cycle arrest and apoptosis in cancer cells, which, thus, became the focus of this study.

Our study demonstrated that 3-hydroxycinnamic acid inhibits cell proliferation and induces apoptosis in a human cervix cancer cell line (HeLa). Alterations in nuclear morphology, laddering fragmentation of DNA and the appearance of hypodiploid cells all indicated that 3-hydroxycinnamic acid induced apoptosis in these cells. The result showed that 10 mM 3-hydroxycinnamic acid induced G0/G1 arrest in HeLa cells after 72 h. It is likely that the toxic effect of high doses of 3-hydroxycinnamic acid may inhibit the enzymes which are associated with the cell cycle. As shown in Figure 3A, B and C, after exposure to a high dose of 3-hydroxycinnamic acid, the treated cell arrested in G0/G1-phase, and sub-G1-phase cells appeared. These results suggested that the cells were blocked at G0/G1-phase and died as a result. Furthermore, the morphology of the cells was changed and the viable cell number and the proliferation of cells were decreased under light-phase microscope examinations, MTT assay and flow cytometry analysis. Apoptosis was also examined by DNA gel electrophoresis for DNA laddering fragmentation and flow cytometry for the sub G1 group in the cell cycle analysis. The onset of cytofluorimetric alterations in the examined cells were in agreement with time- and dose-dependent biochemical indicators of apoptosis. We also examined the checkpoint enzyme for S-phase which led to G2/M, showing that cyclin B was increased. It is generally accepted that normal cells and some tumor cells, when growing exponentially in culture, enter the S-phase of the cell cycle with maximal cyclin E expression (12). In fact, a minimal cyclin E threshold level is often seen, as the cells with cyclin E expression below this threshold would not enter the S-phase (12).
Figure 2. Morphological changes of human cervix HeLa cells in response to 3-hydroxycinnamic acid. HeLa cells were treated with varying concentrations of 3-hydroxycinnamic acid for 24 h. The cells were examined under contrast phase microscope and photographed.

Figure 3. Effects of 3-hydroxycinnamic acid on the HeLa cell cycle and apoptosis (sub-G₁ group). HeLa cells were cultured with 5 or 10 mM 3-hydroxycinnamic acid for various periods, and the cells were harvested and analyzed for cell cycle and sub-G₁ group (panel A: control and 5 mM 3-hydroxycinnamic acid). The percent of HeLa cells in phases (panel B) and apoptosis (panel C) were evaluated by flow cytometry, as described in Materials and Methods. Data represents mean ± S.D. of three experiments. *p<0.05
p53, caspase 3 and Bax were increased. Apoptosis was p53- and caspase 3- pathway-dependent from the Western blotting methods. Bcl-2 family members are the regulators of apoptosis. Bcl-2 is an intracellular suppressor of apoptosis and, thus, serves a cyto-protective function in cells (13) and functions by heterodimerizing with its pro-apoptotic relative Bax (14). Our data showed that 3-hydroxycinnamic acid increased the levels of Bax. Caspase-3 is a cysteine protease that exists as an inactive zymogen in cells and which is activated by proteolytic events, which lead to the active form (15). 4-Hydroxycinnamic acid inhibited lipid peroxidation more significantly than 3-hydroxycinnamic acid, and this effect was related to free radical formation (16). Therefore, we report here that 3-hydroxycinnamic acid-induced apoptosis in cervix cancer cells in vitro is dependent of the presence of p53. The other interesting point is that the cellular level of p53 could dictate the response of the cells, because lower levels of p53 are prone to cell cycle arrest, whereas higher levels of p53 result in apoptosis (17). However, the increasing levels of wild-type p53 may, in part, account for the high susceptibility to apoptosis in response to anticancer drugs. It has been reported that p53 plays an important role in transcriptional activation when DNA damage leads to apoptosis (18, 19).

Taken together, both the morphological changes and the levels of caspase-3 from HeLa cells after treatment with 3-hydroxycinnamic acid suggest a caspase-3-dependent apoptotic pathway. This is also confirmed by the flow cytometric analysis showing the occurrence of the sub-G1 group. The importance of these findings is that we have shown the occurrence of apoptosis of HeLa cells after treatment with 3-hydroxycinnamic acid via two distinct mechanisms: caspase-dependent apoptosis and p53-dependent apoptosis. These findings provide important new insights into the possible molecular mechanism of the anticancer activity of 3-hydroxycinnamic acid.

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References


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