Ellagic Acid Induced p53/p21 Expression, G1 Arrest and Apoptosis in Human Bladder Cancer T24 Cells

TE-MAO LI¹, GUANG-WEI CHEN¹, CHIN-CHENG SU², JAUNG-GUNG LIN¹, CHIN-CHUNG YEH³, KWORK-CHU CHENG⁴ and JING-GUNG CHUNG⁵

¹Schools of Chinese Medicine, ²Department of Microbiology, China Medical University, Taichung City 400, Taiwan; Departments of ³Surgery and ⁴Urology, China Medical University Hospital, Taichung City 400, Taiwan; ⁵Department of Surgery, Jen-Ai Hospital, No 483, Tong-Rong Road, Tali, Taichung, Taiwan, R.O.C.

Abstract. It is well known that dietary phenolic compounds can elicit cellular responses such as cytotoxicity, cell cycle arrest and apoptosis by activating a cascade of molecular events. Ellagic acid is one of these phenolic compounds, but the exact mechanism of its action is still unclear. The objective of this study was to investigate ellagic acid-induced cell cycle arrest and apoptosis in T24 human bladder cancer cells in vitro. Assays were performed to determine cell viability, cell cycle arrest, apoptosis, caspases-3 activity and gene expression, measured by flow cytometric assay, polymerase chain reaction (PCR) and determination of caspase-3 activity. Ellagic acid significantly reduced the viable cells, induced G0/G1-phase arrest of the cell cycle and apoptosis. Ellagic acid also increased p53 and p21 and decreased CDK2 gene expression, that may lead to the G0/G1 arrest of T24 cells. Ellagic acid also promoted caspase-3 activity after exposure for 1, 3, 6, 12 and 24 h, which led to induction of apoptosis. Furthermore, the ellagic acid-induced apoptosis on T24 cells was blocked by the broad-spectrum caspase inhibitor (z-VAD-fmk).

The "People's Health Bureau of Taiwan" reported that about 3.47/100,000 people die annually of bladder cancer in Taiwan. The most frequently used clinical therapeutic treatments are radiotherapy, chemotherapy and surgery. However, these strategies for treatment of human bladder cancer are not totally satisfactory. Many compounds purified from plants have shown anticancer activity, such as taxol (1) and camptothecin (2), both of which can induce cancer cells to differentiate and undergo apoptosis. Furthermore, some anticancer drugs are known to induce apoptosis via the inhibition of topoisomerase II and activation of caspase-3 activity (2-5).

Ellagic acid is a phenolic compound present in fruits and nuts. It has been demonstrated that ellagic acid can inhibit tumor growth caused by chemical carcinogens in an animal model (6-10). It had also been reported that ellagic acid inhibits TPA-induced ornithine decarboxylase activity, N-acetyltransferase activity, hydroperoxide production and DNA synthesis (11-16). Further, it was reported that ellagic acid induced G0/G1 arrest and apoptosis in human cervical carcinoma Ca Ski cells (17). Recently, it was demonstrated that ellagic acid induced cell cycle arrest in human colon cancer cells resulting in the down-regulation of IGF-II (18). However, there are no reports that address whether ellagic acid affects cell cycle arrest and apoptosis of human bladder cancer T24 cells. This study showed that ellagic acid induced G0/G1 arrest through increased p53 level and induced apoptosis through activation of caspase-3 activity in human bladder cancer T24 cells.

Materials and Methods

Chemicals and reagents. Ellagic acid, Tris-HCl, triton X-100, ribonuclease-A, trypsin blue and propidium iodide (PI) 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). McCoy's 5a medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was from Boehringer Mannheim (Mannheim, Germany). Human bladder cancer cell line. The human bladder cancer cell line (T24: human; female; 81 years old) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in 75-cm² tissue culture flasks and grown at 37°C in a humidified 5% CO₂ and 95% air atmosphere in McCoy's
Cell viability was determined by using flow cytometry. T24 cells were plated in 12-well plates at a density of 5x10⁵ cells/well and grown for 24 h. Then, various concentrations of ellagic acid (0, 1, 5, 10, 25, and 50 μM) were added and the cells were grown for different periods of time. DMSO (solvent) was used for the control regimen. For determining cell viability, the flow cytometric protocol was used, as previously described (19).

Flow cytometry analysis of DNA content for cell cycle and apoptosis analysis in T24 cells co-treated with different concentrations of ellagic acid. About 5x10⁵ T24 cells/well in 12-well plate were incubated with ellagic acid (0, 1, 5, 10, 25 and 50 μM) for different time periods before the cells were harvested by centrifugation. 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 (Sigma) and 0.1% triton x-100. After 30 min at 37°C in the dark, the cells were analyzed with 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 (20).

Caspase activity determination. T24 cells were plated in 12 well plates at a density of 5x10⁵ cells/well with various concentrations of ellagic acid using only DMSO as a solvent for the control regimen. The cells were then incubated at 37°C in a humidified 5% CO₂ chamber for 12 h. About 5x10⁵ cells (T24) were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5mM EDTA, 10 mM Tris-HCl, pH 8.0, 2mM dithiothreitol, 1 mM PMSF, 1 μg/ml aprotinin, 1 mg/ml leupeptin) for 30 min at 4°C followed by centrifugation at 10,000 xg for 30 min. For caspase-3 activity determination, 50 μL reaction mixtures with fluorogenic report substrate peptides were used. The substrate peptide (200 μM) was incubated at 37°C with cytosolic extracts (15 μg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, 0.1% 3-[3-chloroamidopropyl]dimethylammonio-1-propanesulfonate). Fluorescence was determined after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent; Labsystems).

Inhibition of ellagic acid-induced apoptosis by the caspase inhibitor z-VAD-fmk in T24 cells. In order to examine whether or not caspase-3 activation was involved in apoptosis triggered by ellagic acid, T24 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to treatment with 25 μM ellagic acid. Apoptosis and caspase activity were then determined as described above.

Reverse transcriptase polymerase chain reaction (RT-PCR) for examining the gene expression of p53, p21, CDK2 and β-actin in T24 cells after exposure to ellagic acid. The total RNA was extracted from T24 cells using Qiagen RNeasy Mini Kit after 24 h cotreatment with different concentrations of ellagic acid, as described previously (14). Total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined in a micro-centrifuge tube to a final volume of 12.5 μl. The entire mixture was heated at 70°C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription were as described in the instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. When amplifying target cDNA, the components in 50 μl of solution were as follows: 1.5 mM MgCl₂, 0.2 mM dNTP mix, 20 pmol of each primer, cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers was as follows: p53: CAGCCAAAGTCTGTGAATGGCAATAC and CTTGTCGAGAAAAGTCTTTCTGACATC p21: AGTGGCAGCGCAGCAGCTGAGA and RTGAAACTCTGCTGATGCTGTTCTG CDK2: GCATTGTTTGGAGGCTGTT and AGAACTGCGCCACCGACT β-actin: GCTCGTGTCGCAACACGGCTC and CAAAACTGACTGTGCTGTCTTC (21).

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

Results

Effects of various concentrations of ellagic acid on cell morphology and viability of human bladder cancer T24 cells. In the presence of ellagic acid (1-50 μM), the cells were collected and stained by propidium iodine and analyzed by flow cytometry. The results indicated that cells were increasingly stained as the time and concentration increased, suggesting that ellagic acid induced cell death on T24 cells. Further increasing the concentration of ellagic acid resulted in increased morphological changes (cell death) and a greater decrease of viable T24 cells (Figures 1, 2 and 3).

Ellagic acid induced cell cycle arrest and apoptosis in human bladder cancer T24 cells. The flow cytometry results for cell cycle analysis indicated that, during the 48 h time period, ellagic 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 typical pattern of DNA content that reflected G0/G1-, S- and G2/M- phases of the cell cycle (Figures. 4A and B). The ellagic acid-treated cells showed a typical 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 Fig. 4A. A pre-G0/G1 apoptotic peak was very clear after cells were treated for 48 h. The percentage of apoptotic cells in various concentrations of ellagic acid are shown in Fig. 4C. An increase of ellagic acid concentration led to an increase of apoptosis in T24 cells.

Effects of ellagic acid on p53, p21 and CDK2 gene expression in intact T24 cells. Changes in p53, p21 and CDK2 mRNA levels in response to varying concentrations of ellagic acid
were studied by PCR. The results from PCR are presented in Figures. 5A, B and C. The data demonstrated that 1-50 µM ellagic acid increased p53 and p21 levels and decreased CDK2 levels. These effects of ellagic acid on p53, p21 and CDK2 were also dose-dependent.

Inhibition of ellagic acid-induced caspase activity and apoptosis by the caspase inhibitor z-VAD-fmk in human bladder tumor T24 cells. These experiments detected ellagic acid induced caspase-3 activity and also examined whether caspase-3 activation was involved in apoptosis triggered by ellagic acid. The data is presented in Figures. 6A and B and indicates that ellagic acid increased caspase-3 activity. This effect was dose- and time-dependent. The caspase inhibitor z-VAD-fmk decreased caspase-3 activity. T24 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to treatment with ellagic acid. When cotreated with ellagic acid and z-VAD-fmk,
inhibition of ellagic acid-mediated caspase-3 activation was accompanied by the marked attenuation of ellagic acid-induced apoptotic cell death (Figures 7A and B). The data also indicated that activation of caspase-3 contributed to ellagic acid-induced apoptosis in T24 cells.

Discussion

In the present work, we investigated the possibility that ellagic acid-induced apoptosis can occur dependently of cell cycle arrest. Our results indicate that ellagic acid was able to induce, in a dose-dependent manner, G1 arrest and apoptotic cell death in T24 cells, as determined by flow cytometric analysis of hypodiploid nuclei. Ellagic acid-induced G1 arrest and apoptosis appeared to be statistically significant at 10, 25 and 50 μM. The mechanisms underlying the ellagic acid-induced G1 arrest and apoptosis of T24 cells were also investigated. Specifically, the critical elements of drug-induced cell cycle arrest and apoptosis, including the checkpoint elements of the cell cycle, activation of caspases and mitochondrial function with concomitant release of apoptogenic factors, were analyzed.

In general, two major pathways are involved in apoptosis: the mitochondrion-initiated pathway and the cell surface death receptor pathway. Both pathways are associated with caspase-3. Apoptosis was induced by activating caspase-3, amongst many other caspases. Caspase-3, one of the downstream effector caspases, works as a key trigger of apoptosis, which eventually results in cell death (22). Evidence indicates that subapoptotic concentrations of cytotoxic drugs can induce growth arrest with senescence features and that p53 and p21WAF-1/CIP-1 are major players in this process (23, 24). We have demonstrated that ellagic acid induced death of the tumor cells mainly by caspase-dependent mechanisms. Inhibition of caspase activity by z-VAD-fmk significantly reduced apoptosis induction by ellagic acid.

Furthermore, in addition to its role as a cell cycle regulator, p21WAF-1/CIP-1 functions to prevent apoptosis, mediating the survival function of phosphatidylinositol through phosphorylation of AKT (24, 25). It is possible that p21WAF-1/CIP-1 functions in protecting DNA-damaged cells from becoming apoptotic, while p53 mediates DNA repair in the process.

In cancer treatment, cell death is an important factor influencing cell number and susceptibility to neoplastic transformation, as well as sensitivity to chemotherapeutic agents. It is well documented that cell death can be distinguished biochemically and morphologically into two distinct types, called necrosis and apoptosis (26). Morphological changes occur in cells undergoing apoptosis, including shrinkage of the cell and DNA fragmentation into membrane-bound apoptotic bodies in which most of the organelles remain intact before being rapidly phagocytosed by neighbouring cells (27). However, the results from agarose gel electrophoresis indicated the present of a ladder pattern of DNA fragments from T24 cells after exposure to ellagic acid (data not shown). Therefore, ellagic acid-triggered apoptosis might be induced in some fractions of the cells existing in the G0/G1-phase, which are committed
to G0/G1 arrest. It is conceivable that cells overexpressing wtp53 will not survive in vivo due to p53-induced G1 arrest and apoptosis (28, 29). It has also been reported that p53 status in tumor cells influence apoptotic decisions in response to hypoxia (30).

The PCR assay demonstrated that ellagic acid promoted p53 and p21 gene expression and inhibited CDK2 gene expression, and therefore may lead to G0/G1 arrest in the cell cycle. Ellagic acid has been demonstrated to exhibit antineoplastic activity, through induction of apoptosis and cell cycle arrest. During the normal cell cycle, the kinase activity of CDK2/cyclin E is elevated specifically at the G0/G1-phase transition and decreases at the onset of anaphase. Many studies have reported that cyclin-dependent kinase 2 (CDK2) and cyclin-E are involved in the cell cycle from G1- into S-phase, and our PCR data showed that ellagic acid inhibited
CDK2, which may be the mechanism resulting in G1/M arrest in the cell cycle of T24 cells. The data from the present study showed that ellagic acid induced apoptosis, since cell death was mediated by caspase-3 activation. Investigators had demonstrated that caspase-3 plays an important role in chemotherapy of HL-60 cells (31) and growth factor withdrawal of hematopoietic cells (32). Apoptosis was completely inhibited by pretreatment with the caspase inhibitor z-VAD-fmk. This phenomenon also showed that the decrease of caspase-3 activity led to an increase in the number of viable T24 cells when treated with ellagic acid. Apparently ellagic acid-induced apoptosis is caspase-3-dependent.

In this study, we reported that ellagic acid induced apoptosis in T24 cells as well as caspase-3 activation.

Figure 4. Effects of ellagic acid on the T24 cell cycle and apoptosis (sub-G1 group). T24 cells were cultured with varying concentrations of ellagic acid for 48 hours, and the cells were harvested and analyzed for cell cycle and sub-G1 group (panel A: control and 1, 5, 10, 25, 50 μM ellagic acid). The percent of T24 cells in phase (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

Figure 5
However, ellagic acid did not affect the activity of caspases-1 and -8 (data not shown). It is well known that the ratio between pro- and anti-apoptotic proteins determines, in part, the susceptibility of cells to a death signal which leads to apoptosis (33-35). Our results indicated that ellagic acid-induced apoptosis in T24 cells was prevented by the presence of catalase (data not shown). Catalase induced photolytic activation of procaspase-3, which led to inhibition of caspase-3 activity, and finally to decrease of the frequency of apoptosis. Interestingly, susceptibility to the apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond by apoptosis.

Figure 5. Representative PCR showing changes in the levels of p21, p53 and CDK2 in human T24 cells after exposure to ellagic acid. T24 cells (5x10^6/ml) were treated with 0, 1, 5, 10, 25 and 50 μM ellagic acid for 24 hours before total RNA was prepared and PCR was performed, as described in Materials and Methods.

Figure 6. Effects of ellagic acid on caspase-3 activity. T24 cells were incubated with various concentrations of ellagic acid for various time periods. Then, the cells were harvested and were analyzed for caspase-3 activity (panel A and B), as described in Materials and Methods. Data represents mean ± S.D. of three experiments. *p<0.05

Figure 7. Effects of ellagic acid on caspase-3 activity and apoptosis. T24 cells were cultured with various concentrations of ellagic acid with or without z-VAD-fmk co-treatment for 48 h. Then, the cells were harvested and were analyzed for caspase-3 activity (panel A) and apoptosis (panel B), as described in Materials and Methods. Data represents mean ± S.D. of three experiments. *p<0.05

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and, thus, defects in the process of apoptosis may be closely associated with carcinogenesis (36). Therefore, multiple mechanisms may be involved in the growth-inhibitory effects of ellagic acid. In the future, studies focusing on cell signaling and the biological significance of ellagic acid-induced apoptosis and cell cycle arrest would elucidate the mechanisms of the chemotherapeutic potency of ellagic acid in human cancer.

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