Antioxidant and Apoptosis-inducing Activities of Ellagic Acid

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Abstract. *Background: Antioxidant, antiproliferative and apoptosis inducing activities of a natural polyphenolic compound, ellagic acid, were studied. Materials and Methods: DPPH radical scavenging and lipid peroxidation inhibitory activities were observed. Activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were measured in ellagic acid-treated V79-4 cells. For apoptotic inducing activity, human osteogenic sarcoma (HOS) cell proliferation, chromosomal DNA degradation and changes in apoptosis-related protein levels were measured. Results: Ellagic acid showed high DPPH radical scavenging and lipid peroxidation inhibition activities. SOD, CAT and GPX activities were significantly increased in ellagic acid-treated V79-4 cells. Ellagic acid significantly reduced HOS cell proliferation, and induced apoptosis evidenced by chromosomal DNA degradation and apoptotic body appearance. Bax expression was induced and caspase-3 was activated by ellagic acid treatment. Conclusion: Ellagic acid exhibited both antioxidant activity in V79-4 cells and apoptosis-inducing activity in HOS cells through the up-regulation of Bax and activation of caspase-3.*

Green tea has been the most highly consumed beverage, along with water, for many centuries in Asian countries. Since the beneficial effects of green tea are known, many studies have focused on the identification of the components of green tea and the investigation of the active ingredients mechanism (1-4). Ellagic acid is a polyphenolic compound present in green tea and other natural sources including pomegranate, strawberries, blackberries, raspberries, walnuts and bark of *Eucalyptus* (3, 5-6). Ellagic acid has been found to have antioxidant, anticarcinogenic, antifibrosis, antiplasmodial activity and chemopreventive activity (3, 6-10).

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It was reported that many antioxidant substances have anticancer or anticarcinogenic properties. For example, resveratrol in grapes and other food products has been shown to protect cells from oxidative damage and death and to prevent carcinogenesis in a murine model (11-13). The present study was, therefore, designed to investigate whether ellagic acid has a protective effect against H_2O_2-induced oxidative stress in Chinese hamster lung fibroblast (V79-4) cells and to characterize the mechanism of its apoptosis-inducing effect in human osteogenic sarcoma (HOS) cells.

Materials and Methods

Chemicals. Ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenyic acid 2,6,2',6'-dilactone) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest analytical grade and were purchased from common sources.

DPPH free radical scavenging activity. In order to measure antioxidant activity, the DPPH free radical scavenging assays were carried out according to previously described procedures (14).

Cell culture and cytotoxicity test. The Chinese hamster lung cell line V79-4 (ATCC CCL-93) was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM glutamine and antibiotics in a humidified incubator with 5% CO_2/95% air at 37˚C. Human osteogenic sarcoma (HOS) cell line (ATCC CRL-1543) was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics. Exponentially growing HOS cells were seeded at $5x10^4$ cells/well in a 96-well plate and treated with the indicated concentrations of ellagic acid or vehicle. After various periods of exposure, the general viability of cultured cells was determined by assaying the reduction of MTT to formazan (15). Experiments were performed at least in triplicate.

Lipid peroxidation inhibitory activity. Lipid peroxidation was determined by measuring malondialdehyde (MDA) (14). V79-4 cells in culture were incubated with ellagic acid at various concentrations (0, 4, 20 and 100 μg/ml) for 60 min, followed by 100 μM H_2O_2 for 60 min. Inhibitory activity against lipid peroxidation was expressed as IC_{50}.

Assays for antioxidant enzymes. V79-4 cells were treated with 0, 4, 20 and 100 μg/ml of ellagic acid for 60 min. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay described

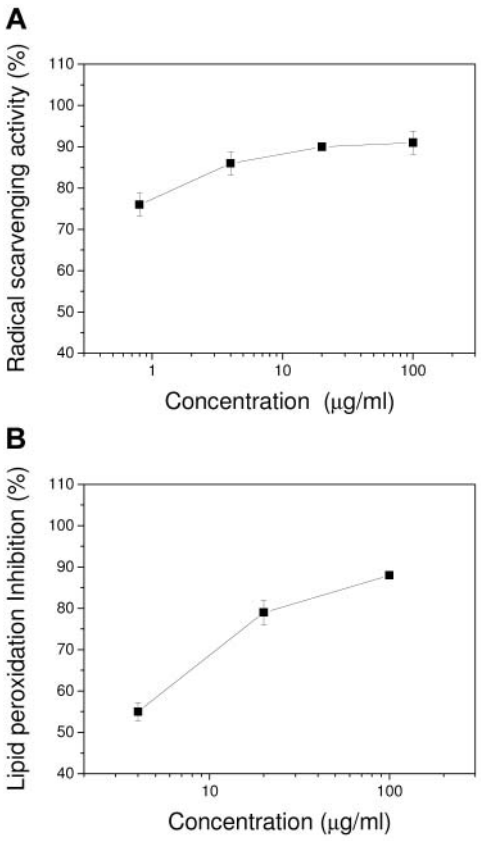

Figure 1. *DPPH radical scavenging (A) and lipid peroxidation inhibition (B) activities of ellagic acid. Each experiment was performed at least 3 times and data are expressed as average percent change from control ± S.D.*

previously (14). The results are expressed as enzyme activity per mg protein, as compared to the corresponding control cultures.

DNA fragmentation analysis. HOS cells were treated for different periods with various concentrations of the sample. Cells were then harvested and DNA fragmentation was analyzed as described elsewhere (16).

Nuclear morphology and flow cytometric analysis. HOS cells were treated with 10 μg/ml of ellagic acid for 0, 24, 48 and 72 h. Morphology of cellular nuclei was observed as described previously (14). Or cells were harvested and used for flow-cytometric analysis as described previously (17).

Western blot analysis. HOS cells were treated with ellagic acid and subjected to Western blot analysis, as described previously (17). Blots were probed with mouse monoclonal anti-human anti-caspase-3 (Transduction Laboratory, USA), and rabbit monoclonal anti-human anti-Bax and anti-PARP (Santa Cruz Biotechnology, USA) antibodies. Immunoreactivity was detected using either an anti-mouse (Santa Cruz Biotechnology) or anti-rabbit (Amersham Biosciences, UK) peroxidase-conjugated secondary IgG antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

Results

DPPH radical scavenging and lipid peroxidation inhibitory activities of ellagic acid. The antioxidant activity of ellagic acid was evaluated on the basis of its DPPH free radical scavenging activity and by the inhibition of lipid peroxidation (Figure 1). The DPPH radical scavenging activity of ellagic acid showed relatively high radical scavenging activity. More than 75% of DPPH radical was scavenged in the range of concentrations used in this study (0.8-100 μg/ml). As shown in Figure 1B, cells pretreated with ellagic acid exhibited a dose-dependent reduction in lipid peroxidation induced in H_2O_2-treated V79-4 cells. Treatment of 4, 20 and 100 μg/ml of ellagic acid inhibited lipid peroxidation by 55%, 79% and 88%, respectively (IC_{50} value <4.0 μg/ml).

Effect of ellagic acid on antioxidant enzyme activity. In order to investigate whether these antioxidant properties of ellagic acid were mediated by an increase in antioxidant enzymes, we measured superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities in V79-4 cells treated with the extract (Figure 2). Treatment with the extract at doses of 4, 20 and 100 μg/ml induced SOD activity to 31.1, 34.9 and 36.6 U/mg protein (25%, 40% and 47% increase), respectively (Figure 2A). The activity of SOD in control cells untreated with the extract was 24.9±1.5 U/mg protein. Ellagic acid also increased CAT activity dose-dependently (Figure 2B). At doses of 4, 20 and 100 μg/ml, the measured CAT activity was 17.2, 18.9 and 22.2 U/mg protein (control value was 14.9±1.9 U/mg protein). The relative increase in CAT activity was 16%, 27% and 49%, respectively. GPX activity increased dramatically in response to treatment with the extract (Figure 2C). This occurred in a dose-dependent manner, in that 4, 20 and 100 μg/ml of ellagic acid increased GPX activity to 15.0, 16.3 and 18.3 U/mg protein (28%, 39% and 56% increase), respectively. The activity of GPX in control cells untreated with the extract was 11.7±1.3 U/mg protein.

Induction of apoptosis by ellagic acid. The anti-proliferative effect of ellagic acid on a cancer cell line, HOS, was evaluated with the MTT assay. When cells were treated for 48 h with 0, 4, 20 and 100 μg/ml of ellagic acid, the relative cell proliferation progressively decreased in a dose-dependent manner, as shown in Figure 3. The IC_{50} value for ellagic acid on HOS cells was 6.5 μg/ml.

To determine whether ellagic acid-meditated inhibition of growth and proliferation was associated with apoptosis, we examined the ellagic acid-induced chromosomal DNA degradation in HOS cells. Analysis of DNA extracted from ellagic acid-treated HOS cells additionally revealed a progressive, time- and dose-dependent increase in

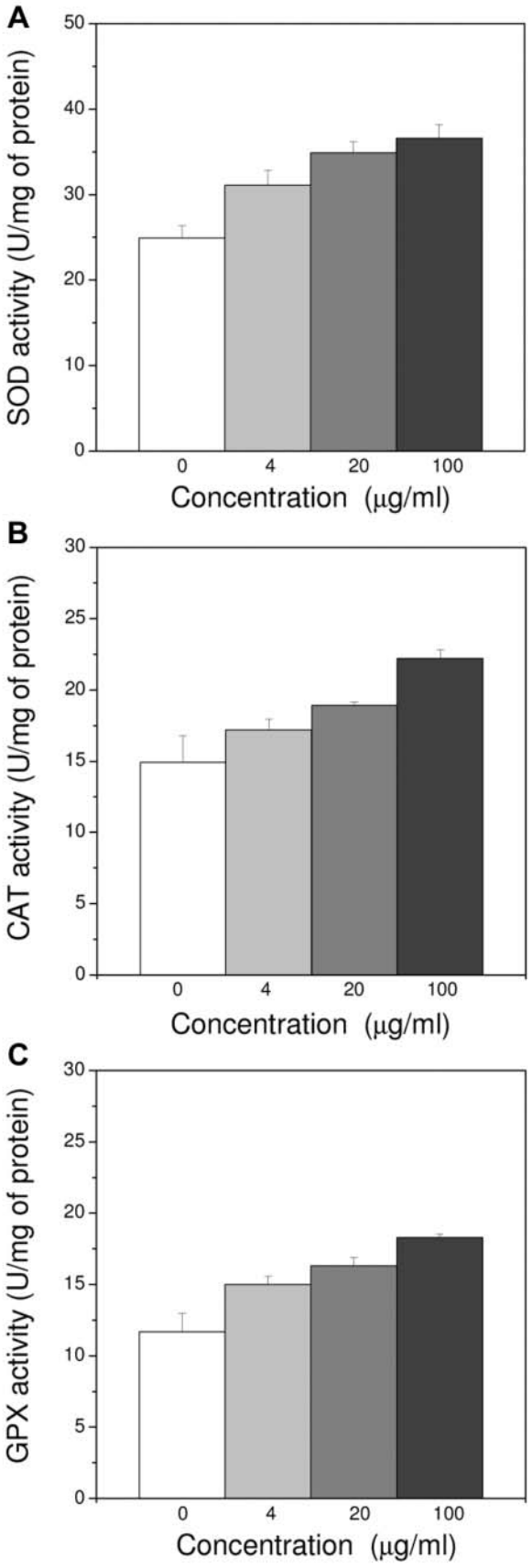

Figure 2. *Effect of ellagic acid on antioxidant enzymes. V79-4 cells were treated with 0, 4, 20 and 100 μg/ml of ellagic acid and superoxide dismutase (SOD) activity (A), catalase (CAT) activity (B) and glutathione peroxidase (GPX) activity (C) were measured as described in Materials and Methods section. Each experiment was performed at least 3 times and the data are expressed as average enzyme units per mg protein from control ± S.D.*

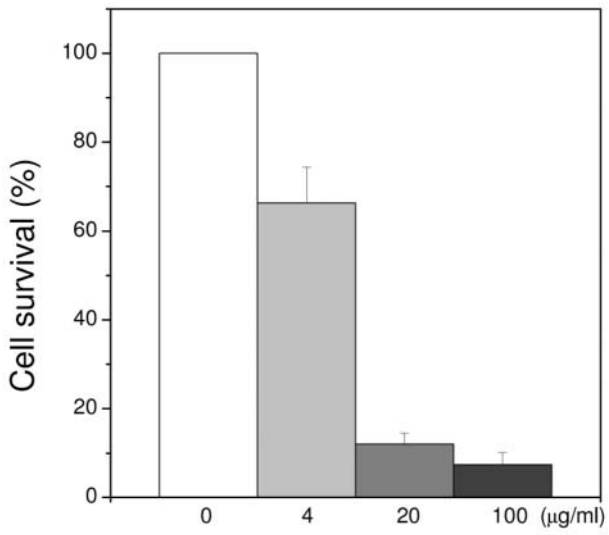

Figure 3. *Antiproliferative activity of ellagic acid in HOS cells. HOS cells were precultured in 96-well microplates for 16 h and then incubated with 4, 20 and 100 μg/ml of ellagic acid for 48 h. Cell survival was measured by the MTT assay. Data are presented as means ± SD. The percentage of cell growth in the control group was designated as 100%.*

chromosomal DNA degradation (Figure 4), which may be associated with the degradation of chromosomal DNA at the linker regions characteristic of apoptotic cells.

Appearance of apoptotic bodies by ellagic acid treatment. In order to analyze the apoptosis induction effect of ellagic acid, we used propidium iodide to stain nuclei of HOS cells treated with ellagic acid. As shown in Figure 5A, control cells exhibited intact nuclei, but cells treated with ellagic acid showed significant time-dependent nuclear fragmentation. The induction of apoptotic bodies in ellagic acid-treated HOS cells was further analyzed by flow-cytometric determination of DNA content. Histograms of DNA content obtained from propidium iodide-stained HOS cells, showed that the percentage of cells with reduced DNA content progressively increased over time. Apoptosis was negligible within 24 h of treatment. Forty-eight and 72 h after ellagic acid treatment (10 μg/ml), however, the percentage of apoptotic cells increased to 15.5 and 22.8%, respectively (Figure 5B). The profile for the ellagic acid-induced increase in hypodiploid DNA content closely correlated with the chromosomal DNA

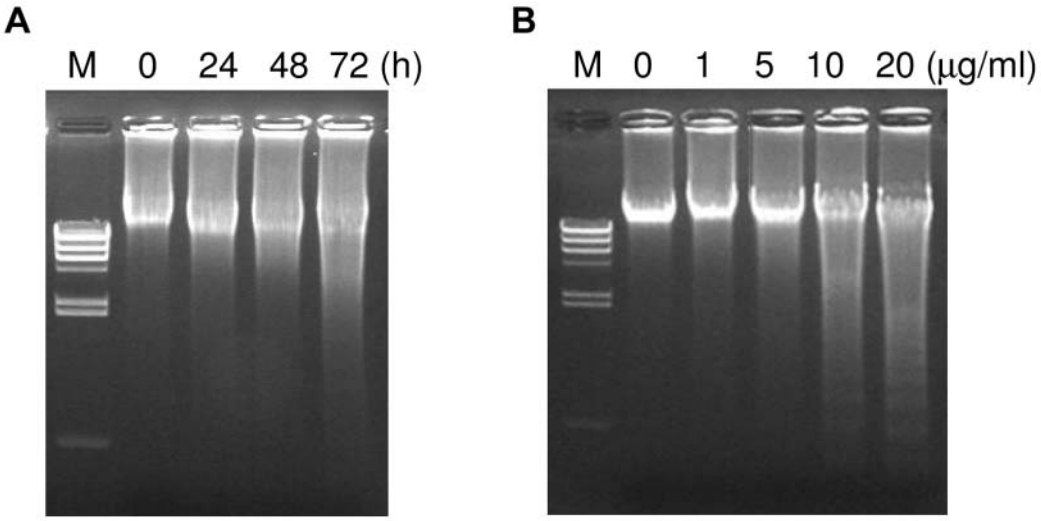

Figure 4. *Ellagic acid induced a time- and dose-dependent induction of chromosomal DNA degradation in HOS cells. (A) HOS cells were treated with ellagic acid at a concentration of 10 μg/ml and harvested at 0, 24, 48 and 72 h. (B) Cells were treated with ellagic acid at concentrations of 0, 1, 5, 10 and 20 μg/ml for 72 h. Chromosomal DNA was extracted and subjected to electrophoresis on 1.5% agarose gels followed by ethidium bromide staining.*

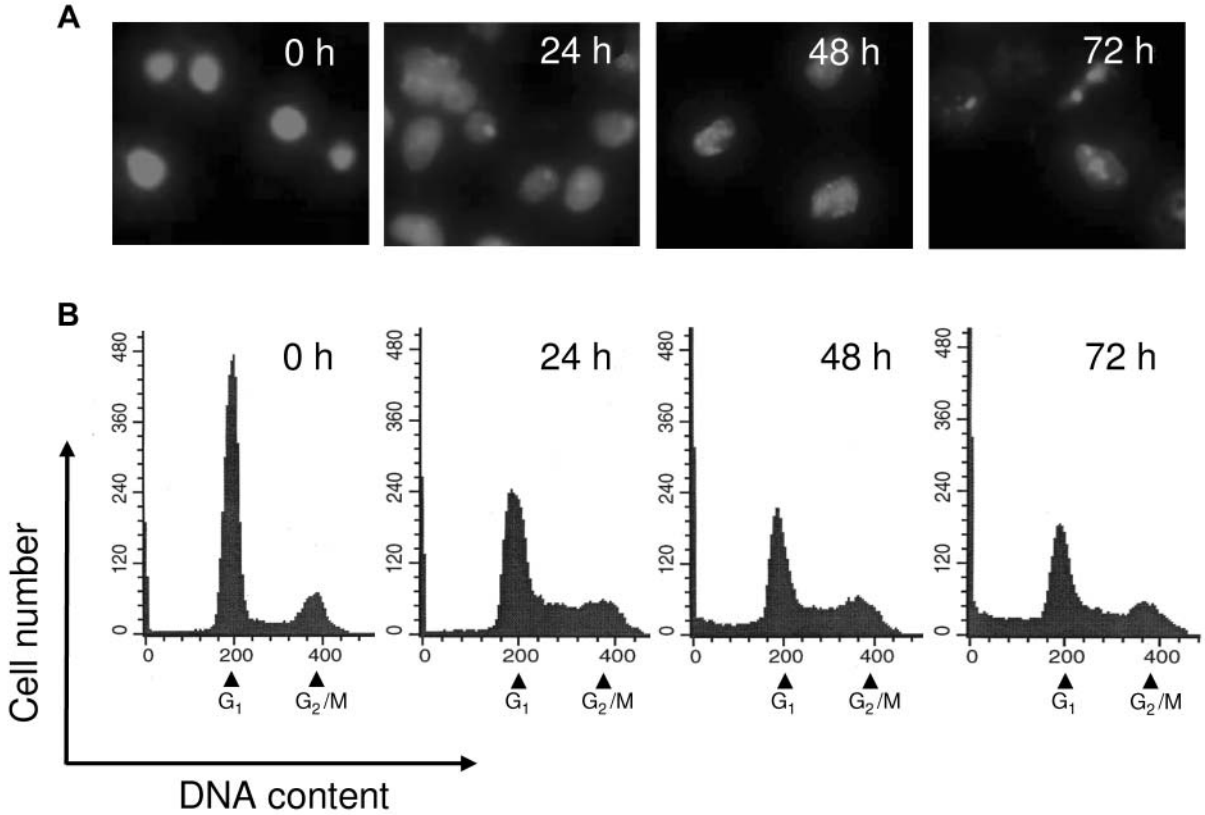

Figure 5. *Apoptotic bodies were induced by ellagic acid treatment. HOS cells were treated with 10 μg/ml of ellagic acid for 0, 24, 48 and 72 h and were stained with propidium iodide. (A) Cellular morphological changes were observed using a fluorescent microscope at the magnitude of 200X. (B) DNA content analysis was performed using a flow cytometer.*

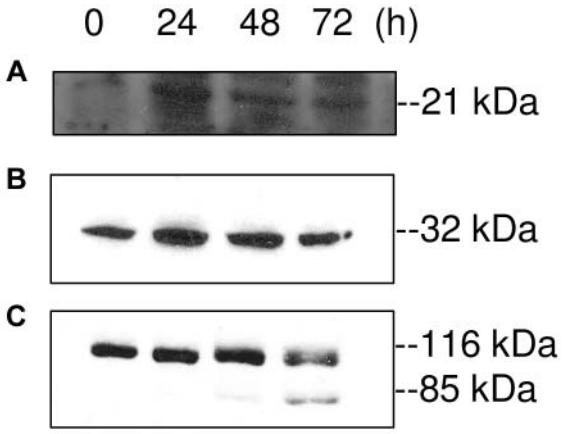

Figure 6. *Changes in the expression of apoptosis-related proteins in response to treatment with ellagic acid. HOS cells were treated with 10 μg/ml of ellagic acid for 0, 24, 48 and 72 h. Cell extracts were subjected to western blotting to determine immunoreactivity levels of Bax (A), procaspase-3 (B), and PARP (C) as described in Methods. Representative western blots are shown.*

degradation. In parallel to the increase in the number of cells with sub-G_1 hypodiploid DNA content, there was a decrease in the number of cells with diploid DNA content.

Effect of ellagic acid treatment on caspase-3 activation. In order to investigate the mechanism by which ellagic acid induce apoptosis, various apoptosis-related proteins were examined. HOS cells were cultured in media containing 10 μg/ml of ellagic acid for 0, 24, 48 and 72 h. At each time-point, total protein was isolated and Bax, caspase-3, and PARP [poly(ADP-ribosyl)polymerase] immunoreactivity levels were measured by Western blotting (Figure 6). The pro-apoptotic protein, Bax was increased in response to ellagic acid treatment (Figure 6A). Pro-caspase-3 levels were also decreased, implying that the levels of active caspase-3 were increased. To investigate the enzymatic activation of caspase-3, we measured the cleavage of PARP, which is a caspase-3 substrate. When cells were treated with 10 μg/ml of ellagic acid, a time-dependent increase in the formation of the 85 kDa fragment and a decrease in the 116 kDa PARP were observed (Figure 6B).

Discussion

In this work, anti-oxidative and apoptosis inducing effect of a polyphenolic compound, ellagic acid, were evaluated. Ellagic acid exhibited significant DPPH free radical scavenging activity and inhibited lipid peroxide production in V79-4 cells exposed to H_2O_2. It was also found that ellagic acid increased the activity of three antioxidant enzymes, SOD, CAT and GPX, which are altered in various diseases involving free radical attack (18). The activities of these enzymes were enhanced in an environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rat brain (19). Other studies also reported anti-oxidative properties of ellagic acid against oxidative stress in PC 12 cells (20).

As stated earlier, several anti-oxidative compounds have been shown to protect against experimental carcinogenesis and to induce apoptosis of cancer cells. Ellagic acid also showed enhanced radiation-induced oxidative stress and cytotoxicity in tumor cells (21). The present results demonstrated that anti-oxidative compound, ellagic acid also induced apoptosis in HOS cells. The induction of apoptotic cell death was accompanied by characteristic morphological and structural changes. Chromosomal DNA fragmentation, as determined by agarose gel electrophoretic analysis and fluorescent microscope observation, were consistent with apoptosis in cells treated with ellagic acid. The flow cytometry data more clearly confirmed apoptosis in ellagic acid-treated HOS cells.

Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. Apoptosis induction mechanism of ellagic acid is not fully understood yet. It is suggested that ellagic acid may reveal anti-proliferative activity through inhibition of inositol-1,4,5-triphosphate 3-kinase, which is known as the best binding target for antiproliferative agents (22). In the present study, it was observed that caspase-3 was activated and the caspase substrate PARP was proteolytically cleaved to low molecular weight fragments in ellagic acid-treated HOS cells. A similar observation reported that ellagic acid interacts with other polyphenolic compounds synergistically in the induction of caspase-3 (23). In addition to caspase-3 activation, the levels of Bax, a pro-apoptotic protein, were increased upon treatment of ellagic acid, resulting in a decrease in the ratio of Bcl-2/Bax, one of the major events that regulate apoptosis. Taken together, these findings suggest that ellagic acid exhibits anti-oxidant activity in a normal cell line and apoptosis induction activity in a cancer cell line, through the down-regulation of Bcl-2/Bax and activation of caspase-3.

Acknowledgements

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