Ellagic Acid, a Dietary Polyphenol, Selectively Cytotoxic to HSC-2 Oral Carcinoma Cells

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Abstract. Background: The antiproliferative and apoptotic effects of ellagic acid, a dietary polyphenol, were studied. Materials and Methods: The neutral red cytotoxicity assay compared the sensitivities of gingival fibroblasts and HSC-2 oral carcinoma cells to ellagic acid. The ferrous ion oxidation xylene orange assay and levels of intracellular reduced glutathione were used to assess pro-oxidant nature of ellagic acid. Antioxidant activity was demonstrated in cells co-treated with H2O2 and ellagic acid by 2',7'-dichlorodihydrofluorescein diacetate staining and in cells co-treated with gallic acid and ellagic acid by morphological analysis. Apoptosis was assessed by microscopy, flow cytometry, luminescence, and immunoblotting. Results: Ellagic acid was cytotoxic to carcinoma cells, but not to normal cells. Its pro-oxidant nature was minimal, whereas its antioxidant property was biologically significant. Ellagic acid-treated cells demonstrated apoptotic morphology, induction of apoptosis (flow cytometry), increase in caspase 3/7 activities (luminescence), and activation of caspase 3 and cleavage of poly ADP ribose polymerase (immunoblot). Conclusion: Ellagic acid exhibited significant antioxidant, but not pro-oxidant, activity and was selectively cytotoxic to oral carcinoma cells.

Epidemiological studies have indicated that diets rich in fruits, vegetables, and nuts promote health and well-being and reduce the risk of various chronic pathologies. Research in cancer prevention, in particular, has benefited tremendously from the discovery that many natural plant products exert anticancer effects. Many of these studies, conducted with human cells in vitro, have evaluated plant extracts or specific phytochemicals for their cancer chemopreventive properties. Plant-based food products contain high levels of a diverse spectrum of phytochemicals, of which the polyphenols are the most significant. This research evaluated the anticancer effects of ellagic acid (Figure 1), common in green tea, grape seed extract, pomegranate, strawberries, blackberries, raspberries, and walnuts. The focus of this research was the oral cavity, as oral cancer is one of the most disfiguring types of cancer, since surgical removal of the tumor may lead to facial disfigurement, and as oral squamous cell carcinoma is the fifth most common type of cancer worldwide (1).

Polyphenols are interesting in that they exhibit both antioxidant and pro-oxidant activities (2), either or both of which may be significant enough to exert biological effects. For example, the cytotoxicity of epigallocatechin gallate, the main polyphenol in green tea, and of theaflavin-3,3’-digallate, the main polyphenol in black tea, correlated with their strong pro-oxidant property (3, 4). Conversely, epicatechin gallate, a green tea polyphenol and a poor generator of oxidants, exerted cytotoxicity that was independent of its pro-oxidant property (5). Although the antioxidant nature of ellagic acid has been noted (6), there is little information on its prooxidant nature and whether it is sufficient to evoke a biologic response from target cells. Using HSC-2 oral carcinoma cells, the pro-oxidant and antioxidant properties of ellagic acid were studied.

In vitro research with ellagic acid has shown its ability to exert growth-inhibiting and apoptosis-inducing cytotoxicity towards cancer cells, including pancreatic (7), breast, prostate gland (8), leukemic (9), neuroblastoma (10), colon (11), osteogenic sarcoma (12), and tongue (13) cells. As far as we know, there are no studies on the comparative responses of human oral cavity carcinoma cells and normal gingival fibroblasts to ellagic acid. The studies presented herein compared the antiproliferative activity of ellagic acid on HSC-2 carcinoma cells isolated from the oral palate to that on normal gingival fibroblasts.

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Hydrogen peroxide (H₂O₂) assay. According to previously described procedures (3-5). To assess viability, the neutral red (NR) assay, concentration of test agent. After exposure to the test agents for one day, the cells were washed with PBS, trypsinized, and diluted to a density just below 5×10⁵ cells/ml. A 20 μl sample of cells, added to 380 μl of Guava ViaCount Reagent (Millipore, Billerica, MA, USA), was placed on ice in the dark for 5 min. Cell viability, apoptosis, and cell death were determined with a Guava EasyCyte Miniflow Cytometer (Millipore). The Guava ViaCount Reagent distinguishes between viable, apoptotic, and nonviable cells based on the differential permeability of its DNA binding dyes. The fluorescence of each dye is resolved operationally to allow for the quantitative assessment of viable, apoptotic, and non-viable cells present in a suspension (15).

**Cells.** HSC-2 Human squamous carcinoma cells derived from the oral cavity were obtained from H. Sakagami, Department of Dental Pharmacy, Meikai University School of Dentistry, Saitama, Japan, and normal human HF-1 fibroblasts from P. Sacks, New York University School of Dentistry, New York, NY, USA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 110 mg/l pyruvate (cat. no. 11995-080), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and 1.25 μg/ml amphotericin B, termed the growth medium, and were maintained in a humidified atmosphere with 5.5% CO₂ at 37°C. Cell dissociation was achieved with 0.1% trypsin-0.04% EDTA for the carcinoma cells and 0.05% trypsin-0.02% EDTA for the fibroblasts.

**Chemicals.** Ellagic acid (Sigma Chemical Co., St. Louis, MO, USA) was solubilized in dimethyl sulfoxide (DMSO), sonicated for three minutes, and prepared fresh daily. The stock concentration of 1% ellagic acid was high enough so that when diluted prior to use, the residual concentration of DMSO was not cytotoxic. For use in the studies, ellagic acid was directly introduced into exposure medium DMEM lacking pyruvate (cat. no. 11995-065), 10% Serum Plus (JRH Biosciences, Lenexa, KS, USA), 2% FBS, and antibiotics, followed by vortexing; fresh solutions were made for each experiment.

**Cell proliferation assay.** Individual wells of a 96-well microtiter tissue culture plate were inoculated with 0.2 ml of the growth medium containing 2×10⁴ cells/well for HSC-2 cells and 1.5×10⁴ cells/well for the HF-1 fibroblasts. After a day of incubation, the growth medium was removed and replaced with exposure medium, with or without ellagic acid at different concentrations. In some studies, the cells were coexposed to ellagic acid and 110 mg/l pyruvate. One complete row, i.e. eight wells, was used for each concentration of test agent. After exposure to the test agents for one to three days, viability was assessed with the neutral red (NR) assay, according to previously described procedures (3-5).

**Hydrogen peroxide (H₂O₂) assay.** Measurement of H₂O₂ was carried out by the ferrous ion oxidation xylenol orange (FOX) method using the PeroXOquant Quantitative Peroxide Assay Kit, lipid-soluble formulation (Pierce Biotechnology, Rockford, IL, USA) as previously described (14).

**Materials and Methods**

**Intracellular glutathione assay.** Cells, maintained in growth medium and grown to about 80% confluence in 60 mm tissue culture plates, were washed with phosphate buffered saline (PBS) and treated for 4 h in serum-free DMEM containing 50, 100, or 150 μM ellagic acid. Three plates of cells were used per concentration of ellagic acid. Measurements of intracellular reduced glutathione (GSH) were performed according to the procedures of Weisburg et al. (3).

**Apoptosis assay: Flow cytometry.** HSC-2 cells, grown to ~80% confluence in 60 mm plates, were untreated or treated for 72 h with different concentrations of ellagic acid. Afterwards, the cells were washed with PBS, trypsinized, and diluted to a density just below 5×10⁵ cells/ml. A 20 μl sample of cells, added to 380 μl of Guava ViaCount Reagent (Millipore, Billerica, MA, USA), was placed on ice in the dark for 5 min. Cell viability, apoptosis, and cell death were determined with a Guava EasyCyte Miniflow Cytometer (Millipore). The Guava ViaCount Reagent distinguishes between viable, apoptotic, and nonviable cells based on the differential permeability of its DNA binding dyes. The fluorescence of each dye is resolved operationally to allow for the quantitative assessment of viable, apoptotic, and non-viable cells present in a suspension (15).

**Apoptosis assay: Western blotting.** HSC-2 cells grown to ~80% confluence were untreated or treated for 48 h with ellagic acid in exposure medium. Cells were lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA), containing complete EDTA-free protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN, USA), and then centrifuged (12,000×g, 10 min) to remove cellular debris. Protein concentrations of the cell lysates were quantified with the BCA Protein Assay Kit (Pierce Biotechnology). Equal concentrations of total protein from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [10% for poly ADP ribose polymerase (PARP) detection; 4-20% for caspase-3 detection] (Thermo Scientific), electroblotted to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA), and blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were then probed with polyclonal antibody to PARP (rabbit antisemum, diluted 1:5,000; Abcam, Cambridge, MA, USA) to detect both full-length and cleaved PARP, or with anti-caspase-3 (rabbit antisemur, Cell Signaling Technology, Danvers, MA, USA). The portion of the membrane corresponding to the molecular weight of actin was probed with a rabbit antibody to actin (rabbit antisemur, diluted 1:7,500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to ensure equal protein loading. Following incubation with the appropriate peroxidase-conjugated secondary antibodies (1:5,000 dilution), membranes were developed using the ECL detection kit (Amersham). Western blot analyses were performed a minimum of three times (4).

**Apoptosis assay: Caspase 3 and 7 activation (luminescence).** HSC-2 cells, seeded into a 96-well white-walled microtiter tissue culture plate, were treated for 48 h with ellagic acid. Cells were lysed and caspase activity was determined by adding the Caspase-Glo 3/7 reagent (Promega, Madison, WI, USA) containing the luminescent caspase-3/7 substrate. Cleavage of the substrate by active caspases present in the cell lysate resulted in a luminescent signal, measured with a Promega Glomax Multi Detection luminometer (Promega, Madison, WI, USA).

**Microscopy.** A: Brightfield microscopy for antioxidant property of ellagic acid. Cells were seeded onto coverslips in 35 mm diameter tissue culture dishes containing growth medium. After the...
monolayers reached 70% confluence, the cells were untreated for 6 h (control) in growth medium or treated for 6 h in medium amended with 33.1 μM ellagic acid, 175 μM gallic acid [a pro-oxidant polyphenol (15)], or a combination of ellagic and gallic acids. Thereafter, the cells were washed in PBS, fixed in methanol, stained with aceto-orcein, and viewed with a brightfield microscope observing changes in cell density and morphology.

B: Fluorescent detection of apoptotic cell morphology. As described above, cells on coverslips were untreated or treated with 175 μM ellagic acid for 48 h, stained with acridine orange (4 μg/ml in PBS), and viewed under a fluorescent microscope observing morphological abnormalities, such as cell blebbing and hypercondensation of nuclei.

C: Fluorescent detection of intracellular reactive oxygen species (ROS). Cells, grown on coverslips until approximately 80% confluence, were washed with PBS and treated with exposure media (serum-free DMEM, without pyruvate and phenol red) amended with different concentrations of ellagic acid, H₂O₂, or a combination of ellagic acid and H₂O₂, for 4 h at 37°C. A 10 mM stock solution of 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) was prepared by dissolving the powder in DMSO and then diluting it with warm exposure medium to 20 μM. HSC-2 cells, washed with PBS, were treated with DCHF-DA for 30 min at 37°C. Thereafter, the extracellular DCHF-DA was removed and the cells were incubated for an additional 30 min at 37°C in the presence of warm exposure medium. During this time, the internalized dye was hydrolyzed by intracellular esterases and the resultant dichlorodihydrofluorescein was oxidized by intracellular ROS to produce a fluorescent product, dichlorofluorescein (DCF). Following this incubation, the cells were washed with PBS, fixed with 3.7% formaldehyde in PBS, air dried, and attached to slides. The slides were observed with a Zeiss microscope equipped with a filter set capable of detecting DCF with an absorption maximum of 492 nm and a fluorescent emission maximum of 517 nm. The intensity of fluorescence, indicative of intracellular ROS, was compared in untreated control cells and in cells treated with ellagic acid alone, H₂O₂ alone, and with a combination of ellagic acid and H₂O₂.

Statistics. All experiments were performed a minimum of three times. Data for the FOX assay are presented as the arithmetic mean±standard errors of the mean (S.E.M.) and those for the cytotoxicity assay are presented as the mean arithmetic percentages relative to the control±S.E.M. Experimental data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey’s multiple range test for significant differences. The p-value of the effect had to be ≤0.05 to be considered significant.

Results

Cytotoxicity assays. Preliminary studies compared the responses of carcinoma HSC-2 cells and normal gingival HF-1 fibroblasts to ellagic acid. Ellagic acid, at concentrations up to 200 μM, was nontoxic to HSC-2 and HF-1 cells after a one-day exposure. At longer exposure times, differential sensitivities to the two cell types were noted. Toxicity towards the HSC-2 cells, but not towards the fibroblasts, was noted after two- and three-day exposures to ellagic acid. For the HSC-2 cells, the midpoint cytotoxicity (NR₅₀) values for two- and three-day exposures to ellagic acid were estimated at 260 and 142 μM, respectively (Figure 2A). The HF-1 cells were resistant to 200 μM ellagic acid for similar exposure times (Figure 2B). As the fibroblasts were unaffected by ellagic acid, at the concentrations and exposure times studied, further studies were directed to HSC-2 cells only.

Pro-oxidant studies. The generation of H₂O₂ in cell culture exposure medium amended with 50 to 200 μM ellagic acid for 4 h was determined with the FOX assay. Although a progressive increase in H₂O₂ was detected as the concentration of ellagic acid was increased, the overall generation of H₂O₂ was relatively minor. For comparative purposes, H₂O₂ was quantified in medium amended with similar concentrations of
gallic acid, another nutraceutical polyphenol but with a strong pro-oxidant property (16) (Figure 3).

To confirm that the level of H₂O₂ generated by ellagic acid was not significant enough to promote oxidative stress, the intracellular level of GSH was monitored in HSC-2 cells treated for 4 h with ellagic acid. The background level in nontreated HSC-2 cells was 33 nmoles of GSH/10⁶ cells and in cells exposed to 50, 100, and 150 μM ellagic acid it was 35, 32, and 32 nmoles of GSH/10⁶ cells, respectively. Pyruvate, a scavenger of H₂O₂ which protects cells from pro-oxidant polyphenols (14), had no protective effect at a concentration of 110 mg/l on ellagic acid up to 200 μM, as determined with the NR assay (data not shown).

**Antioxidant studies.** To ascertain the antioxidant property of ellagic acid, DCHF-DA was used to monitor intracellular ROS in HSC-2 cells exposed for 4 h with ellagic acid. The background level of intracellular ROS in nontreated HSC-2 cells was 33 nmoles of GSH/10⁶ cells and in cells exposed to 50, 100, and 150 μM ellagic acid, it was 35, 32, and 32 nmoles of GSH/10⁶ cells, respectively. Pyruvate, a scavenger of H₂O₂ which protects cells from pro-oxidant polyphenols (14), had no protective effect at a concentration of 110 mg/l on ellagic acid up to 200 μM, as determined with the NR assay (data not shown).

To further evaluate the antioxidant nature of ellagic acid, HSC-2 cells were exposed for 6 h to 33.1 μM ellagic acid alone, to a cytotoxic concentration of gallic acid alone, i.e. 125 μM, and to a combination of ellagic acid and gallic acid. The cytotoxicity of gallic acid was correlated to its pro-oxidant nature (17). No differences in cell density and morphology were noted in cells exposed to ellagic acid as compared to untreated control cells. A lesser cell density and aberrant cell morphologies were noted upon treatment with gallic acid. However, as compared to the control, cell density and cell morphology were unaffected in samples co-treated with gallic and ellagic acids (Figure 4C-F).

**Discussion**

Many studies with human cells in culture have evaluated the potential anticancer effects of dietary plant-derived polyphenols, showing that the polyphenols, acting as pro-oxidants, generate sufficient levels of ROS to induce oxidative stress, leading to apoptosis of carcinoma cells. Studies, albeit limited, that compared carcinoma to normal cells showed the normal cells to be less sensitive to pro-oxidant polyphenols than the cancer cells (3, 4, 15). Sakagami in reviewing research on the comparative responses of oral carcinoma cells and gingival fibroblasts to natural plant-derived products concluded that the apoptosis-inducing activity of these test agents, many of which were polyphenols, reflected an undefined tumor-specific activity against oral squamous carcinoma cells (1). As many of these polyphenols exerted pro-oxidant activity, at a level significant enough to induce oxidative stress, a suggested explanation was that the greater sensitivity of cancer cells than of normal cells to pro-oxidant polyphenols reflected their compromised cellular redox status and deficient antioxidant defenses (3, 4, 15). As noted in the FOX assay, ellagic acid was a weak generator of H₂O₂. In the HSC-2 cell-based assays, the level of pro-oxidants produced by ellagic acid was inefficient to reduce the intracellular level of GSH, the main cellular defense against oxidative stress, and co-treatment with pyruvate, a
scavenger of H$_2$O$_2$, had no effect on the cytotoxicity of ellagic acid. Apparently, the differential sensitivity of HSC-2 and HF-1 cells to ellagic acid was not a function of their differential intracellular defensives towards oxidative stress. However, Han et al. showed Chinese hamster lung V79-4 cells treated with ellagic acid exhibited elevated activities of superoxide dismutase, catalase, and glutathione peroxidase (12). Yet no evidence of ellagic acid acting as a pro-oxidant, i.e. as the stimulant to activate these antioxidant activities, was presented. Perhaps the low level of H$_2$O$_2$ generated by ellagic acid as shown in the studies herein with the FOX assay was sufficient to activate the antioxidant enzymatic activities in the V79-4

Figure 4. Antioxidant property of ellagic acid. HSC-2 cells were stained with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min following a 4 h exposure to 200 μM H$_2$O$_2$ (A) or 200 μM H$_2$O$_2$ in the presence of 33.1 μM ellagic acid (B). Fluorescence is indicative of intracellular ROS. The absence of intracellular fluorescence was noted in untreated (control) HSC-2 cells and in HSC-2 cells treated with 33.1 μM ellagic acid alone. HSC-2 cells were untreated (C), and treated for 6 h with 33.1 μM ellagic acid (D), 125 μM gallic acid (E), or a combination of ellagic acid and gallic acid (F). Cells were stained with aceto-orcein and viewed with a brightfield microscope. Magnification, ×320.
Figure 5. A: Cytometric analysis, using Guava ViaCount Reagent, of a 72 h exposure of HSC-2 cells to increasing concentrations of ellagic acid. The percentage of viable, apoptotic, and dead cells were quantified by the flow cytometer. The data are expressed as the arithmetic mean percentage±S.E.M. of viable, apoptotic, and dead cells; *p≤0.05 and **p≤0.01 as compared to untreated cells. B: Representative FACS profiles of the viability and apoptosis of HSC-2 cells after a 72 h treatment with different concentrations of ellagic acid. Viable cells are located on the left side of each panel; apoptotic cells between the two lines; and dead cells on the right side of the panel.
Another approach to explain the differential sensitivities was provided by Losso et al. (8), who noted that several human cancer cell lines, including Caco-2 (colon), MCF-7 and Hs 578T (both breast), and DU 145 (prostate gland), were more sensitive to ellagic acid than were normal human HEL-299 lung fibroblasts. Their research suggested that this differential sensitivity was attributed to a reduction of ATP specifically in ellagic acid-stressed cancer cells, but not in normal fibroblasts.

Intracellular ROS in HSC-2 cells treated with H$_2$O$_2$ were eliminated in the presence of ellagic acid. Similarly, Festa et al. (6) observed similar findings with Chinese hamster ovary cells co-treated with H$_2$O$_2$ and ellagic acid. Furthermore, using the alkaline Comet assay, Festa et al. (6) noted that DNA damage in Chinese hamster ovary cells treated with either H$_2$O$_2$ or bleomycin, a generator of ROS, was markedly reduced upon cotreatment with ellagic acid. Han et al. observed that ellagic acid inhibited lipid peroxidation in Chinese hamster lung V79-4 fibroblasts treated with H$_2$O$_2$ and, in a cell-free assay, showed that ellagic acid exhibited 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity (12). The cytotoxicity of gallic acid, a strong pro-oxidant (16), was correlated to its pro-oxidant nature (17). The cytotoxicity of gallic acid towards HSC-2 cells was lessened in the presence of a non-cytotoxic concentration of ellagic acid. Pavlica and
Gebhardt similarly noted that the cytotoxicity of \( H_2O_2 \) and of \( t \)-butyl hydroperoxide to neuronal PC12 cells was suppressed by ellagic acid (18).

Induction of apoptosis in HSC-2 cells exposed to ellagic acid was shown by various apoptosis-indicators, including the appearance of apoptotic cell morphology, flow cytometry to differentiate viable, apoptotic, and dead cells, activation of caspase-3/7 shown by luminescence; and immunoblot detection of the conversion of procaspase-3 to activated caspase-3 and of the cleavage of PARP, a substrate of caspase-3. The proapoptotic characteristic of ellagic acid has been noted by others and have included other indicators of apoptosis, such as internucleosomal chromosomal DNA degradation (7, 10, 12, 13), stimulation of the mitochondrial apoptotic pathway associated with mitochondrial depolarization, cytochrome c release, and downstream caspase activation (7); effects on signaling pathways, including increased levels of Bcl-2-associated X protein, a proapoptotic protein (12) and inhibition of nuclear factor \( \kappa B \), an anti-apoptotic protein (7); and by blockage of apoptosis by the pan-caspase inhibitor, Z-VAD-FMK (9). Other molecular aspects of ellagic acid cytotoxicity include cell growth arrest at the S and G2/M phases of the cell cycle (11), reduced levels of pro-matrix metalloproteinases (8), and alteration of the expression levels of mitogen-activated protein kinases signaling genes, specifically leading to differentiation of human leukemia HL-60 cells. Int J Hematol 92: 136-143, 2010.

In summary, this research demonstrated that HSC-2 carcinoma cells were more sensitive to ellagic acid than were normal gingival fibroblasts. Regarding the HSC-2 cells, it was also shown that the pro-oxidant nature of ellagic acid was not significant enough to induce oxidative stress, that the antioxidant property of ellagic acid sequestered intracellular ROS in cells challenged with \( H_2O_2 \) and protected against challenge with gallic acid, a pro-oxidant polyphenol, and that ellagic acid induced apoptosis.

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References


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