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In vitro anti-proliferative activities of ellagic acid

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Abstract

The potential cytotoxic and anti-proliferative activities of ellagic acid (a naturally occurring bioactive compound in berries, grapes, and nuts) was evaluated using human umbilical vein endothelial cells (HUVEC), normal human lung fibroblast cells HEL 299, Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 human prostatic cancer cells. Ellagic acid at concentration in the range 10–100 μmol/L did not affect the viability of normal fibroblast cells during a 24-hour incubation. An increase in adenosine triphosphate (ATP) bioluminescence of approximately 18–21% was observed in normal cells incubated with ellagic acid. In contrast, ellagic acid at 1–100 μmol/L dose-dependently inhibited HUVEC tube formation and proliferation on a reconstituted extracellular matrix and showed strong anti-proliferative activity against the colon, breast, and prostatic cancer cell lines investigated. The most sensitive cells were the Caco-2, and the most resistant were the breast cancer cells. Ellagic acid induced cancer cell death by apoptosis as shown by the microscopic examination of cell gross morphology. Ellagic acid induced reduced cancer cell viability as shown by decreased ATP levels of the cancer cells. After 24 hours incubation of 100 μmol/L of ellagic acid with Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells, ellagic acid suppressed fetal bovine serum (FBS) stimulation of cell migration. The apoptosis induction was accompanied by a decreased in the levels of pro-matrix metalloproteinase-2 (pro-MMP-2 or gelatinase A), pro-matrix metalloproteinase-9 (pro-MMP-9 or gelatinase B), and vascular endothelial growth factor ($VEGF_{165}$) in conditioned media. The results suggest that ellagic acid expressed a selective cytotoxicity and anti-proliferative activity, and induced apoptosis in Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells without any toxic effect on the viability of normal human lung fibroblast cells. It was also observed that the mechanism of apoptosis induction in ellagic acid–treated cancer cells was associated with decreased ATP production, which is crucial for the viability of cancer cells.

Keywords: Ellagic acid; Angiogenesis; Human lung fibroblast cells; Caco-2; MCF-7; Hs 578T; DU 145 cancer cells; HUVEC; Metalloproteinase-2 (MMP-2); Metalloproteinase-9 (MMP-9)

1. Introduction

Angiogenesis or the formation of new blood vessels from pre-existing vascular ones has been identified and recognized by various investigations as a therapeutic approach to slow or treat neoplastic and non-neoplastic degenerative diseases; these include cancer, arthritis, diabetes retinopathy, and many others [1–4]. Angiogenesis is a multistep process that involves the contribution of several stimulators. Enzymatic catalysis is a process crucial to the onset and progression of angiogenesis. The potential advantages of regulating early stages of angiogenesis are significant and may prevent the development of complications such as metastasis associated with the progression of the angiogenic process. Vascular endothelial growth factor (VEGF) has been recognized as major stimulator of both physiological and pathological angiogenesis and plays multiple roles in a variety of pathological angiogenesis. VEGF stimulates blood vessel growth and increases vascular permeability. Other important growth factors that complement the action of VEGF include the fibroblast growth factor (FGF), which stimulates endothelial cell proliferation, induces neovascularization in vivo, and is important for tumor survival and maintenance [1]. VEGF is considered as the major soluble mediator of both normal and pathological angiogenesis, and acts through its receptor tyrosine kinases. VEGF exists in four isoforms; of these, the shorter forms $VEGF_{121}$ and $VEGF_{165}$ are the predominant soluble forms, whereas the $VEGF_{189}$ and $VEGF_{206}$ are bound to the extracellular matrix [5]. The soluble forms of VEGF have been detected in serum of cancer patients, and the over-expression of these substances has been correlated with poor prognosis [6]. Therefore, inhibition of VEGF expression is of particular

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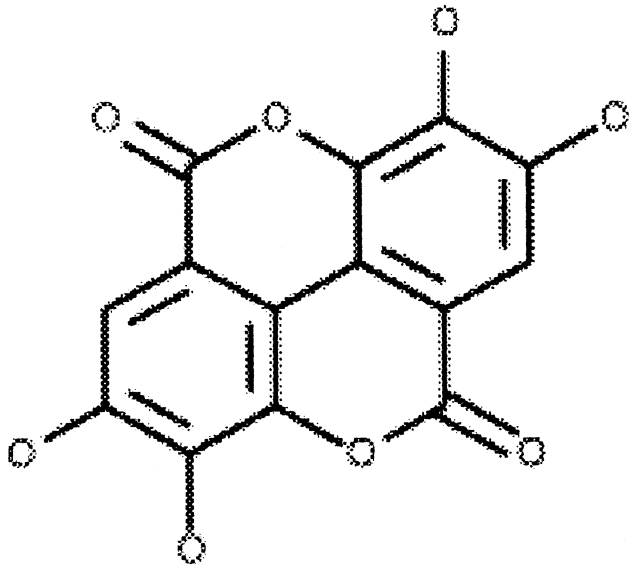


Fig. 1. Structure of ellagic acid.

interest because the onset and/or progression of the metastasis of several human cancers are associated with overexpression of VEGF, among other growth factors.

Ellagic acid (4,4′,5,5′6,6′-hexahydroxydiphenic acid 2,6,2′6′-dilactone) (Fig. 1) is a dimeric derivative of gallic acid found in woody plants, berries, grapes, and nuts [7]. Ellagic acid is found in muscadine grapes at average concentrations of 5.6 mg per 100 g of fresh muscadine grape wine and 10.2 mg per 100 g of fresh muscadine grape juice, and has been associated with the formation of insoluble sediments during muscadine wine production [7,8]. Important biological activities such as radical scavenging activities, chemopreventive, and antiviral activities have been ascribed to ellagic acid [9]. This study was undertaken to evaluate the potential inhibitory activity of ellagic acid against cancer cell proliferation and possibly angiogenesis.

2. Methods and materials

2.1. Cell treatments

HUVEC cells were obtained from and grown as per ATCC procedures. Kaighn's F12K media plus 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement and 10% fetal bovine serum (FBS) was used. Cells were passaged once per week at a 1:2 split and refed once per week. Cancer cells (Caco-2, MCF-7, Hs 578T, and DU 145), and normal human lung fibroblast cells (HEL 299) were purchased from the American Type Culture Collection (Manassas, VA) and cultured as per ATCC instructions. Cell viability estimated by trypan blue exclusion test was between 90% and 95%.

2.2. In vitro angiogenesis assay

The CHEMICON In Vitro Angiogenesis Assay Kit (catalog no. ECM 625 from Chemicon International, Inc., Temecula, CA) was used. Briefly, after preparing the gel matrix and allowing it to solution, 1.8×10^5 HUVEC cells, alone or mixed with various concentrations of ellagic acid (1–100 μmol/L dissolved in 0.5% DMSO) was seeded per well onto the surface of the polymerized EC matrix as per the manufacturer's suggestions. The plate was incubated at 37°C for 6 hours. Tube formation was inspected and photographed using a Leitz phase contrast inverted microscope.

2.3. Cell cytotoxicity and proliferation assay

Normal and cancer cells at 1.8×10^5 cells/well were allowed to attach overnight at 37°C and 5% CO_2. The culture medium was then aspirated, and fresh culture medium containing either the vehicle (0.5% DMSO) or ellagic acid (1–100 μmol/L) was added to each well and incubated for 6 hours. After 6 hours, the cells were trypsinized, counted using trypan blue exclusion, and subcultured at a density of 3500 cells/cm^2. Cell populations were monitored for 72 hours, and cultured was terminated and regarded as senescent when the cell population did not increase in 72 hours. The cytotoxicity of ellagic acid against normal and cancer cells was also measured using the ViaLight HS (BioWhittaker Laboratory, Rockland, ME) luciferin and luciferase-based assay using adenosine triphosphate (ATP) bioluminescence as marker of cell viability. The test provides an estimation of mitochondrial activity. Cells that had been treated with 0–100 μmol/L of ellagic acid for 6 hours and untreated cells (normal human lung fibroblast cells HEL 299, Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells) were plated at a density of 1.8×10^5 cells in 100μL per well in 96-well plates. Nucleotide (ATP) releasing reagent (100 μL) was added to each well and the plate was incubated for 10 minutes at room temperature. Cell lysate (180 μL) was transferred to a luminescene compatible plate. The 96-well plates were read using a Perkin-Elmer LS 50B luminometer (Perkin-Elmer, Norwalk, CT). ATP levels in cells were normalized to levels in untreated control cultures. To characterize the mode of cell death, gross morphology using microscopic examination was assessed. Experiments were performed in triplicate.

2.4. Quantitative cell migration assay

Cancer cell chemotaxis toward 10% FBS was tested in the presence of 100 μmol/L of ellagic acid using the Chemicon Quantitative Cell Migration (QCM™, catalog number ECM 510) Assay. Briefly, aliquots of cancer cells (2.5×10^5 cells in 100 μl) were placed into the migration chamber. Serum free media (150 μL) either alone as control or in the presence of 10% FBS as chemoattractant was added to the wells of the lower chamber. The plate was covered and

incubated for 24 hours at 37°C in a 5% CO_2 incubator. The migration chamber was placed onto a new lower chamber containing 150 μL of cell detachment buffer to dislodge the cells from underside. Then 50 μL of lysis buffer/dye solution was added to each well of the lower chamber containing 150 μL of cell detachment buffer with the cells that migrated through the membrane. After 15 minutes incubation at room temperature, 150 μL of the mixture was transferred to a new 96-well plate suitable for fluorescence measurement and the fluorescence was read using a Perkin-Elmer LS 50B (Perkin-Elmer, Norwalk, CT) using a 480/520-nm filter set.

2.5. MMP-2/MMP-9 gelatinase assay

Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 prostatic cancer cells were incubated in serum-free medium for 6 hours with 0–100 μmol/L of ellagic acid. The activity of gelatinase A and B (MMP-2 and MMP-9, respectively) secreted into the cell culture media was determined using the CHEMICON Gelatinase Activity Assay Kit ECM700 (Chemicon, Temecula, CA). The assay uses a biotinylated gelatin that is cleaved by active MMP-2 and MMP-9 enzymes. The remaining biotinylated gelatin fragments were then added to a biotin-binding 96-well plate; the addition of streptavidin-enzyme conjugate allowed colorimetric detection of the remaining biotin at 450 nm. A 70-μL quantity of culture medium, for each control or ellagic acid treated cells, was added to 20 μL of 250 mmol/L Tris containing 50 mmol/L $CaCl_2$ at pH 7.5, followed by the addition of 10 μL of *p*-aminophenylmercuric acetate (APMA) from a stock of 20 mmol/L APMA as suggested by the manufacturer. Samples were incubated for 90 minutes (for MMP-2 activity) and 300 minutes (for MMP-9 activity). Activated samples in 20-μL quantities were used for the determination of the activity of MMP-2 and MMP-9. Values of OD_{450} nm obtained with the MMPs in the culture medium using the CHEMICON MMP Gelatinase Activity Kit were compared with OD_{450} nm of positive control MMP-2 and MMP-9 and reported as percentage of control.

2.6. VEGF quantification by ELISA

The *ChemKine* Human Vascular Endothelial Growth Factor (VEGF) Enzyme Immunoassay was used to quantitatively measure $VEGF_{165}$ production in normal human lung fibroblast cells, Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 prostatic cancer cells supernatant. Cancer cells were incubated in serum-free medium or in serum-free medium containing 10–100 μmol/L of ellagic acid. After 24 hours of incubation at 37°C and 5% CO_2, the media were collected and $VEGF_{165}$ was measured by ELISA using the *ChemKine* protocol according to the manufacturer's instructions.

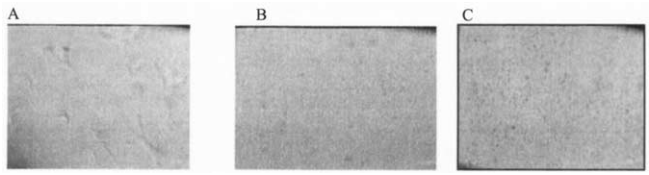


Fig. 2. In vitro angiogenesis assay. After preparing the gel matrix and allowing it to solution, 1.8×10^5 of human umbilical endothelial cells (HUVEC), alone or mixed with 1–100 μmol/L of ellagic acid, were seeded per well onto the surface of the polymerized EC matrix as per the manufacturer's suggestions. The presence or absence of tube formation was observed under a phase contrast inverted microscope. (*a*) Tube formation by HUVEC in absence of ellagic acid; (*b*) HUVEC cells treated with 10 μmol/L; (*c*) HUVEC cells exposed to 100 μmol/L ellagic acid and shows complete disruption of the tube network.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used for significant differences. Statistical significance was defined at $P < 0.05$. Data are presented as mean ± SD.

3. Results and discussion

3.1. In vitro angiogenesis assay, cell proliferation, and migration assays

The ability of ellagic acid to inhibit the growth of HUVEC was assessed. In the absence of ellagic acid, tube formation was observed in the gel matrix containing HUVEC cells (Fig. 2a). A 6-hour exposure to 1–100 μmol/L inhibited the growth of HUVEC with an IC_{50} value of <20 μmol/L. Figures 2b and 2c show the disappearance of tube formation in the presence of 10 and 100 μmol/L of ellagic acid, respectively. As shown in Figs. 3a and 3b, ellagic acid did not affect the viability of normal human fibroblast cells incubated with 100 μmol/L ellagic acid for 24 hours incubation, trypsinized, and subcultured for 72 hours. Ellagic acid at 1–100 μmol/L incubated with cancer cells dose-dependently suppressed cell growth (Figs. 3c–3e). Similar morphological changes were observed when MCF-7, Hs 578T, and DU 145 cancer cells were incubated with increasing concentrations of ellagic acid (Figs. 3f–3k).

An increase of about 18–21% in ATP bioluminescence was observed when normal cells were incubated with 1–100 μmol/L of ellagic acid (Fig. 4). An increase in ATP was also considered as an indirect indicator of increased mitochondrial activity. In cancer cells exposed to 1–100 μmol/L ellagic acid, ATP levels decreased rapidly to <50% after 24 hours, but remained constant at 48 hours incubation (not shown). A decrease in ATP was associated with a decrease in cancer cell viability as shown by the morphological changes of cells exposed to increasing concentration of ellagic acid. Ellagic acid showed strong antiproliferative activity against Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells. Caco-2 cells were the most sensitive cells (IC

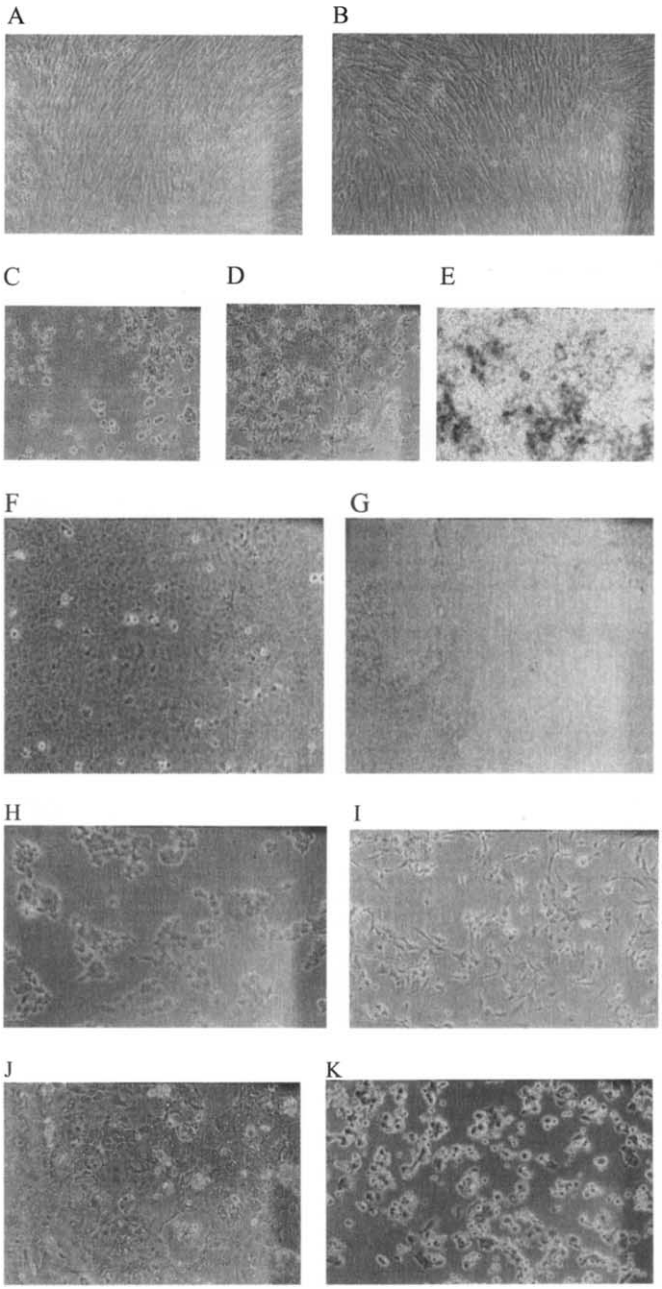


Fig. 3. Cell viability assay. Normal human lung fibroblast HEL 299 cells and cancer cells were incubated in absence or presence of increasing concentrations of ellagic acid. Cell gross morphology was visualized by using an inverted phase microscope (*a–k*). (*a, b:*) normal cell control and normal cells treated with 100 μmol/L ellagic acid; (*c–e*): Caco-2 cells control, and cells treated with 50 and 100 μmol/L ellagic acid, respectively; (*f, g*): MCF-7 control and cells incubated with 100 μmol/L ellagic acid; (*h, i*): Hs 578T control and cells treated with 100 μmol/L ellagic acid; (*j, k*): DU 145 control and cells treated with 100 μmol/L ellagic acid.

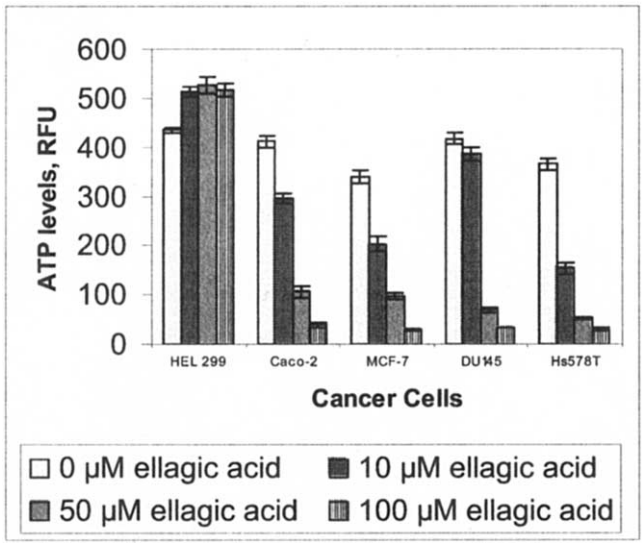


Fig. 4. Cell proliferation assay. Normal and cancer cells were incubated with increasing concentrations (0–100 μmol/L) of ellagic acid. The cells were treated with lysis buffer and the concentration of ATP in the cells was measured using the ViaLight Assay kit. ATP bioluminescence was calculated as percentage of increase or decrease in ATP levels in comparison with ATP luminescence of control (no ellagic acid treatment). ATP values are mean ± SD of three determinations.

$_{50}$ = 37 μmol/L) followed by DU 145 (IC_{50}= 42 μmol/L), Hs 578T (IC_{50}= 59 μmol/L), and MCF-7 (IC_{50}= 72 μmol/L). Microscopic examination of cell gross morphology indicated that cancer cells died by apoptosis. Apoptosis results in the disappearance of cellular integrity, nuclear condensation, and DNA fragmentation. Apoptotic cancer cells were condensed with fragmented nucleus and disintegrated membrane. Ellagic acid–induced apoptotic cervical carcinoma cell death has been reported by Narayanan et al. [10].

Bioluminescence detection of ATP using luciferase is based on the principle that ATP is present in all metabolically active cells. The concentration of ATP can be linearly associated with the amount of light generated when ATP is converted to AMP in the presence of luciferase. The ATP concentration of a cell can be directly related to the cell metabolic activity, as cell injury or death is associated with reduced ATP activity. The bioluminescent detection of ATP is a highly sensitive, simple, and reproducible assay [11–13]. Bruggisser et al. [14] reported that the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay regularly used to measure cell viability and proliferation showed increased MTT formozan formation when cell number (determined by crystal violet staining) was decreased in the presence kaempferol. These observations suggest that the ATP assay may be a better approach for use with phenolic compounds such as ellagic acid and others. The ATP assay takes <30 minutes to perform.

Cancer cells chemotaxis toward 10% FBS as chemoattractant was tested in the presence of 100 μmol/L of ellagic (Fig. 5). Fluorescent measurements taken at 24 hours after incubation indicated that ellagic acid suppressed cell migration in the order Caco-2 > DU 145 > Hs 578T > MCF-7, with Caco-2 cells being the less migrated and MCF-7 the most migrated. However, in general, ellagic acid suppressed the migration of all cancer cells investigated.

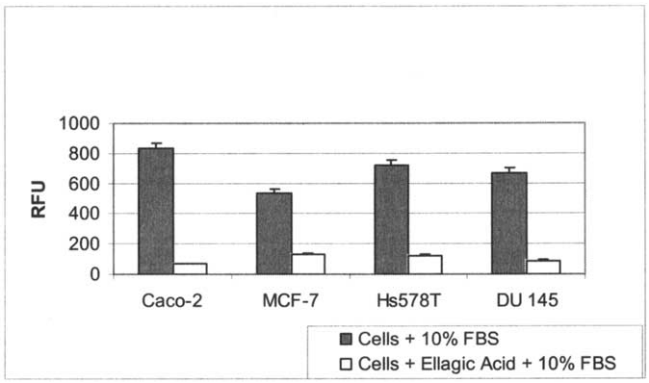


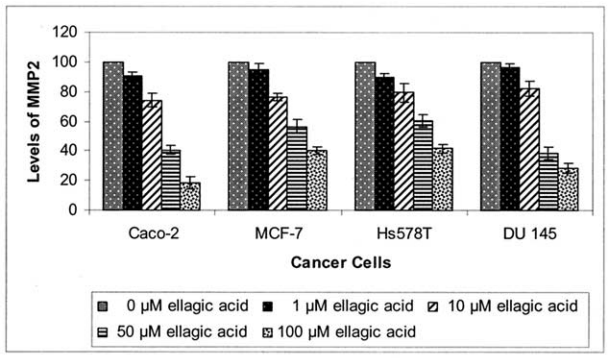
Fig. 5. Quantitative chemotaxis cell migration assay. Cancer cells chemotaxis toward 10% FBS was tested in the presence or absence of 100 μmol/L of ellagic acid and 2.5×10^4 cells per well. Fluorescence measurements were taken at 24 hours post incubation using 480/520 nm filter set.

3.2. MMP-2/MMP-9 gelatinase assay

We tested the effect of ellagic acid on the activity of MMP-2 and MMP-9 in Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 prostatic cancer cells. It is established that most MMPs are secreted as zymogens and are activated extracellularly. Culture media were activated with APMA followed by the use of the CHEMICON MMP Gelatinase Activity Assay Kit to determine the activity of MMP-2 and MMP-9 secreted by ellagic acid treated cells. Figure 6 shows that within 24 hours, the levels of active MMP-2 and MMP-9 secreted into the culture medium decreased as the ellagic acid concentration increased. Ellagic acid at 1 and 10 μmol/L was not very effective against MMP activities. At 50 μmol/L of ellagic acid, MMP-2 and MMP-9 activities of all cells were less than 50% of the control. Higher concentrations of ellagic acid (100 μmol/L) suppressed the metalloproteinase activities of most cells to less than 40% of the control. Mertens-Talcott et al. [8] reported that the physiological concentration of ellagic in the blood plasma, after food ingestion, may be around 5–10 μmol/L, although this estimation was based on other phenolics such as quercetin. At 10 μmol/L, ellagic acid was effective against the MOLT-4 human leukemic cell line within 48 hours [8]. At the molecular level, angiogenesis inhibition includes, among many stimulators, the inhibition of MMP-2 and MMP-9 activities.

3.3. VEGF ELISA assay

Ellagic acid treatment of Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 prostatic cancer cells for 24 hours decreased the secreted $VEGF_{165}$ in conditioned medium in a concentration-dependent manner (Fig. 7). Low levels of $VEGF_{165}$ were measured at 100 μmol/L of ellagic acid and Caco-2 cells were the most affected, followed by DU 145, Hs 578T, and MCF-7. Angiogenesis is increased in



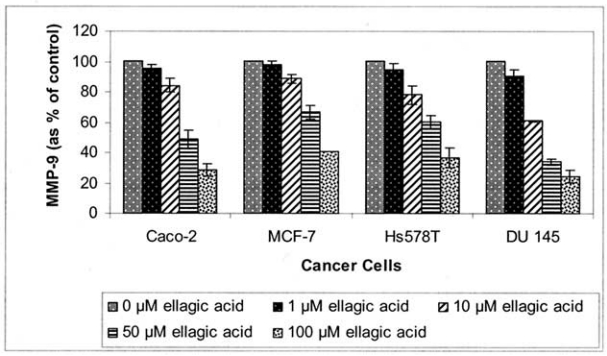


Fig. 6. Inhibitory effect of ellagic acid on the concentration of MMP-2/MMP-9 secreted into the medium of Caco-2 colon, MCF-7 breast, Hs 578T breast, and DU 145 prostatic cancer cells. Cells were incubated at 37°C for 24 hours in the presence of a 0–100 μM of ellagic acid. MMP-2/MMP-9 in the conditioned media was determined using the collagenase assay kit from Chemicon (Temecula, CA). (*a*) Inhibitory activity of ellagic acid against MMP-2;(*b*) inhibitory activity of ellagic acid against MMP-9.

various human cancers, and correlates with tumor progression and metastasis. VEGF has been shown to be a key regulator of angiogenesis necessary for the development of new tumor vessels. Naturally occurring inhibitors of the VEGF receptor tyrosine kinases, such as genistein and curcumin, have shown anti-tumoral and anti-angiogenic activity in several in vitro and in vivo models [15,16]. Ellagic acid suppressed cell proliferation and migration. Low values of ATP correlated with reduced cell growth and enhanced apoptosis; as a result, the expression of MMP-2, MMP-9, and VEGF in the supernatant was reduced. In recent years, there has been debate as to whether serum levels of vascular endothelial growth factor (VEGF) may provide useful prognostic information in patients with various types of cancers [16]. Platelets have been suspected to be the storage units for VEGF because most VEGF in the serum is released from platelets during clotting. Poon et al. [17] used ELISA and real-time quantitative reverse transcription–polymerase chain reaction to measure serum and tumor cytosolic $VEGF_{165}$ level, and tumor $VEGF_{(165)}$ mRNA, respectively, in 60 patients with hepatocellular car-

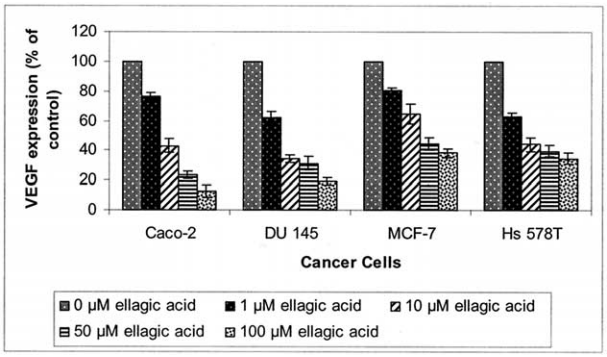


Fig. 7. $VEGF_{165}$ concentration (as percentage of control) in conditioned medium obtained after treatment of cancer cells with 0–100 μmol/L ellagic acid.

cinoma. They found a significant positive correlation between serum $VEGF_{165}$ and platelet counts, a significant correlation between serum $VEGF_{165}$ and tumor cytosolic $VEGF_{165}$ level, and a correlation with $VEGF(_{165})$ mRNA expression in the tumors. Studies by Poon et al. [16] and others [18–21] have provided strong evidence that supports the use of serum $VEGF_{165}$ level as an indirect estimate of tumor VEGF expression. Work is in progress in our laboratory to determine the in vivo effectiveness of ellagic acid against serum $VEGF_{165}$ level. Although cell cycle was not determined in this study, cell proliferation was significantly reduced as shown by the result of ATP levels. The reduced ATP levels may be an irreversible process; and the cells, unable to repair the damages, ultimately undergo processes that lead to reduced VEGF expression and possibly suppression of angiogenesis.

Ellagic acid, a dilactone of hexahydroxydiphenic acid, occurs naturally in berries and nuts such as the raspberry, strawberry, walnut, and pecan in the form of hydrolyzable tannins referred to as ellagitannins [7,22]. Ellagic acid is insoluble at pH <7 but remains soluble at physiological pH. At pH >7, ellagic acid chelates divalent cations such as Zn^{2+}, Ca^{2+}, Fe^{2+}, Cd^{2+}, Cu^{2+}, and others; and this property enhances ellagic acid ability to inactivate metalloproteinases such as MMP-1, MMP-2, MMP-9, and others. Chelation of Zn^{2+} and Cu^{2+} has been associated with antiangiogenic activity [23–26]. The cytotoxicity and anti-proliferative activity of ellagic acid against cancer cells was detected at a concentration range that did not affect normal cell viability. The induction of apoptosis in cancer cells is of substantial interest, because the prevalence of ellagic acid in the daily diet and the insensitivity of normal cells to ellagic acid suggest further investigation into the use of ellagic acid as a potential disease-preventive compound. The findings of this work suggest that ellagic acid can inhibit cancer cell growth, and effect that is likely to be mediated by regulating matrix metalloproteinases, vascular endothelial growth factor expression, and by inducing apoptosis.

Acknowledgment

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References

[1] Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
[2] O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat Med 1996;2:689–92.
[3] Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249–57.
[4] Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.
[5] Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 1991;266:11947–54.
[6] Karayiannakis AJ, Syrigos KN, Polychronidis A, Zbar A, Kouraklis G, Simopoulos C, Karatzas G. Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery. Ann Surg 2002;236:37–42.
[7] Talcott ST, Lee J-H. Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. J Agric Food Chem 2002;50:3186–92.
[8] Mertens-Talcott SU, Talcott ST, Percival SS. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. J Nutr 2003;133:2669–74.
[9] Xu Y, Deng JZ, Ma J, Chen SN, Marshall R, Jones SH, Johnson RK, Hecht SM. DNA damaging activity of ellagic acid derivatives. Bioorg Med Chem 2003;11:1593–6.
[10] Narayanan BA, Geoffroy O, Willingham MC, Re GG, Nixon DW. p53/p21(WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Lett 1999; 136:215–21.
[11] Slater K. Cytotoxicity tests for high-throughput drug discovery. Curr Opin Biotechnol 2001;12:70–4.
[12] Petty RD, Sutherland LA, Hunter EM, Cree IA. Comparison of MTT and ATP-based assays for the measurement of viable cell number. J Biolumin Chemilumin 1995;10:29–34.
[13] Crouch SP, Kozlowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods 1993;160:81–8.
[14] Bruggisser R, von Daeniken K, Jundt G, Schaffner W, Tullberg-Reinert H. Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. Planta Med 2002;68:445–8.
[15] Sengupta S, Gherardi E, Sellers LA, Wood JM, Sasisekharan R, Fan TP. Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. Arterioscler Thromb Vasc Biol 2003;23:69–75.
[16] Shih SC, Claffey KP. Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. Growth Factors 2001;19: 19–34.
[17] Poon RT, Lau CP, Cheung ST, Yu WC, Fan ST. Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma. Cancer Res 2003;63:3121–6.
[18] Pasieka Z, Stepien H, Komorowski J, Kolomecki K, Kuzdak K. Evaluation of the levels of bFGF, VEGF, sICAM-1, and sVCAM-1 in serum of patients with thyroid cancer. Recent Results Cancer Res 2003;162:189–94.
[19] Nishimura R, Nagao R, Miyayama H, Matsuda M, Baba K, Yamashita H, Fukuda M. Higher plasma vascular endothelial growth

factor levels correlate with menopause, overexpression of p53, and recurrence of breast cancer. Breast Cancer 2003;10:120–8.

[20] Riedel F, Gotte K, Li M, Hormann K, Grandis JR. Abrogation of VEGF expression in human head and neck squamous cell carcinoma decreases angiogenic activity in vitro and in vivo. Int J Oncol 2003; 23:577–83.

[21] Cascinu S, Graziano F, Catalano V, Barni S, Giordani P, Baldelli AM, Staccioli MP, Rossi C, Brenna A, Valenti A, Muretto P, Catalano G. Vascular endothelial growth factor and p53 expressions in liver and abdominal metastases from colon cancer. Tumour Biol 2003;24:77–81.

[22] Mullen W, Yokota T, Lean ME, Crozier A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS. Phytochemistry 2003;64:617–24.

[23] Pan Q, Kleer CG, van Golen KL, Irani J, Bottema KM, Bias C, De Carvalho M, Mesri EA, Robins DM, Dick RD, Brewer GJ, Merajver SD. Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. Cancer Res 2002;62:4854–59.

[24] Sartippour MR, Heber D, Zhang L, Beatty P, Elashoff D, Elashoff R, Go VL, Brooks MN. Inhibition of fibroblast growth factors by green tea. Int J Oncol 2002;3:487–91.

[25] Camphausen K, Sproull M, Tantama S, Sankineni S, Scott T, Menard C, Coleman CN, Brechbiel MW. Evaluation of copper chelation agents as anti-angiogenic therapy. Bioorg Med Chem 2003;11:4287–93.

[26] Shian S-G, Kao Y-R, Wu FY, Wu C-W. Inhibition of invasion and angiogenesis by zinc-chelating agent disulfiram. Mol Pharmacol 2003;64:1076–84.