

RESEARCH ARTICLE

Ellagic Acid Inhibits Migration and Invasion by Prostate Cancer Cell Lines

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Abstract

Polyphenolic compounds from pomegranate fruit extracts (PFEs) have been reported to possess antiproliferative, pro-apoptotic, anti-inflammatory and anti-invasion effects in prostate and other cancers. However, the mechanisms responsible for the inhibition of cancer invasion remain to be clarified. In the present study, we investigated anti-invasive effects of ellagic acid (EA) in androgen-independent human (PC-3) and rat (PLS10) prostate cancer cell lines *in vitro*. The results indicated that non-toxic concentrations of EA significantly inhibited the motility and invasion of cells examined in migration and invasion assays. The EA treatment slightly decreased secretion of matrix metalloproteinase (MMP)-2 but not MMP-9 from both cell lines. We further found that EA significantly reduced proteolytic activity of collagenase/gelatinase secreted from the PLS-10 cell line. Collagenase IV activity was also concentration-dependently inhibited by EA. These results demonstrated that EA has an ability to inhibit invasive potential of prostate cancer cells through action on protease activity.

Key words: Ellagic acid - cancer - invasion - migration - metastasis

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Introduction

Prostate cancer is the most common male malignant tumor in the Western countries (Gronberg, 2003). The 5-year survival rate for the localized disease is close to 90% to 100%, while just approximately 32% for the metastatic prostate cancer (Jemal et al., 2008). The major cause of deaths is due to the complications resulting from cancer metastases to distant organs in the body, and treatment of such metastatic disease is one of the major therapeutic challenges. Prostate cancer growth is initially androgen-dependent, medical or surgical castration has been the standard treatment for metastatic prostate cancer. However, the effect of hormonal therapy is temporary, and most tumors become androgen refractory, which obstacle in the treatment of metastatic prostate cancer.

Cancer metastasis is an important aspect necessary for tumor development, proceeds through a multi-step process, including cellular adhesion and invasion through the basement membrane. The ability of infiltration allows malignant cells to invade other organs through the blood stream or lymph vessels (Fidler and Kripke,

1977; Nagase and Woessner, 1999). Therefore, the prevention of tumor metastasis is one of the goals for cancer patients, and cytotoxic agents have been applied in tumor metastasis therapies. However, such therapy has many serious side effects that could diminish the quality of life in cancer patients (Braun-Falco et al., 2006). Recently, many efforts have been made to reduce the spread of malignant tumors; focused on cell invasion using substances in dietary and medicinal plants, because non- or low-cytotoxic agents are required for tumor metastasis therapy (Yodkeeree et al., 2008; Lin et al., 2009; Pitchakarn et al., 2010).

Ellagic acid (EA) is a polyphenolic compound and present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries. It has anticarcinogenic, antioxidant and antifibrosis properties (Mukhtar et al., 1988; Thresiamma and Kuttan, 1996; Stoner and Gupta, 2001; Han et al., 2006). The anti-carcinogenic effect of EA was shown in several cancer types including esophageal, colon, skin, breast and prostate cancers (Stoner and Gupta, 2001; Larrosa et al., 2006; Bell and Hawthorne, 2008; Strati et al., 2009).

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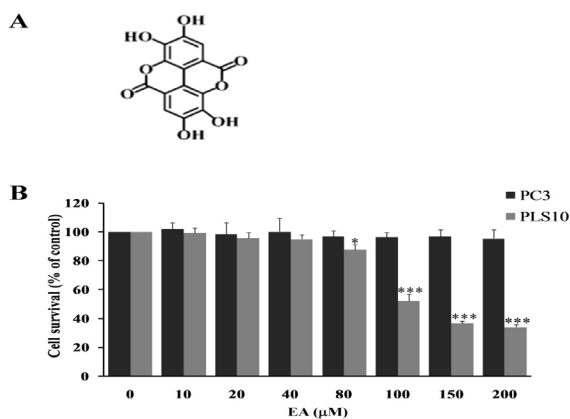


Figure 1. The Cell Growth Effect of EA on PC3 and PLS10. A) The structure of EA. B) The cells were incubated with various concentration of EA for 48 h and then the cell growth was assessed by WST-1 assay. The results are expressed as a percentage of the untreated cell (0 μM). *, ***significantly different from 0 μM at $p < 0.05$ and 0.001 , respectively

It was found that EA could be detected in plasma and accumulated in the intestine and prostate (Larrosa, Tomas-Barberan and Espin, 2006; Seeram et al., 2006; 2007). The amount of information available on the properties and roles of pomegranate extracts including EA is very limited, their potential as chemopreventive and chemotherapeutic agents of cancers needs to be achieved. The present study, we investigated the effect of EA on the invasion of androgen-independent human (PC3) and rat (PLS10) prostate cancer cell lines.

Materials and Methods

Chemicals and cell culture

Ellagic acid, which structure is presented in Fig. 1A, was purchased from Fluka Co.Ltd., Germany. Androgen-independent prostate cancer cell lines, PC-3 (human) which purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA) and PLS10 (rat) that has been established from 3,2'-dimethyl-4-aminobiphenyl plus testosterone-induced carcinomas in the dorsolateral prostate of male F344 rats (Nakanishi, 1996), were used in this study. The cells were cultured in Roswell Park Memorial Institute-1640 Medium (RPMI 1640, Gibco, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS, Life Technologies Japan Ltd., Japan), 50 U/ml penicillin and 50 μg/ml streptomycin, maintained in a humidified incubator with an atmosphere comprising 95% air and 5% CO₂ at 37°C. When the cells reach 70-80% confluence, they were harvested and plated either for subsequent passages or for treatments.

Cytotoxicity and growth inhibition assay

The cells were plated at 2.5×10^3 cells per well in 96-well plates. After 24 h, various concentrations of EA were added into the wells. The cells were incubated for 48 h at 37°C, then cell growth were assessed by soluble

formazan formation, WST-1 colorimetric assay (Roche, Mannheim Germany) (Hamasaki et al., 1996). In each experiment, determinations were carried out in triplicate.

Invasion and migration assay

The cells were seeded on BD Biocoat™ Matrigel™ invasion or migration (BD Falcon insert) chambers (BD Biosciences, Qume Drive San Jose, California USA), with or without EA at indicated concentrations (25 and 50 μM), and incubated at 37°C. For PC3 cells, cells were incubated for 24h, and 10 μg/ml of fibronectin was used as a chemoattractant. For PLS10 cells, cells were incubated for 48h, and 5% fetal bovine serum was used as a chemoattractant. For migration assay, the cells were treated with EA at the concentration of 0, 25 and 50 μM, and incubated for 24 h. Ten μg/ml of fibronectin or 5% fetal bovine serum were used as a chemoattractants for PC3 or PLS10 cells, respectively. The invading or migrating cells were fixed with 100% ethanol for 5 minutes, then stained with 0.5% crystal violet in 20% methanol for 30 minutes and determined areas by ImageJ 1.410 (National Institute of Mental Health, Bethesda Maryland, USA).

Gelatin zymography

The cells were maintained in serum free RPMI 1640 for 24 h then treated with various concentrations of EA. MMP-2 and MMP-9 secretions from PC3 and PLS10 in the conditioned medium were detected by gelatin zymography as previously described (Fernandez-Patron et al., 1999). The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel containing 0.1 mg/ml of gelatin (Bio-Rad Laboratories, Hercules, California USA) under non-reducing condition. After electrophoresis, SDS in the gel was washed twice with 2.5% Triton-X 100 then incubated at 37°C in the incubating buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, and 10 mmol/L CaCl₂, pH 7.4) for 24 h. After incubation, the gel was stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories) in 50% methanol/10% acetic acid, and destained with 10% acetic acid/50% methanol. The bands of gelatinolytic activity were analyzed using ImageJ 1.410.

Measurement of purified collagenase IV and collagenase/gelatinase secreted from PLS10 activities

An EnzChek Gelatinase/Collagenase Assay Kit (Life Technologies Japan Ltd., Tokyo, Japan.) was used for measuring gelatinase/collagenase activity. PLS10 at 106 cells were cultured in FBS-free DMEM for 24 hours, and then the culture supernatant was collected. The substrate, DQTM fluorescein-conjugate gelatin, was incubated with culture medium of PLS10 or collagenase type IV from *Clostridium histolyticum* with EA (0, 25, 50 and 100 μg/ml) for 1.5 h and fluorescence signal representing proteolytic activity was measured using a fluorescence microplate reader (480/530 nm).

Statistical analysis

All experiments were done at least in triplicate to see the reproducibility. All data presented mean±S.D. Statistical comparisons were performed with one-way ANOVA followed by the Dunnett's test. Statistical significance was concluded with $p < 0.05$.

Results

Effect of EA on the cell growth of PC3 and PLS10 cells

WST-1 assay showed that EA treatment caused the reduction of cell growth of PLS10 with inhibitory concentration (IC) 50 about 100 μM while the treatment of EA up to 200 μM did not affect the cell growth of PC3 (Figure 1B). The range of non-toxic concentration was applied in all subsequent experiments.

EA inhibited the invasion and migration of the prostate cancer cells

The invading cells in chambers were presented in Figure 2A which indicates that EA treatment inhibited the invasion of PC3 and PLS10 cells. The inhibition rate in PC3 was approximately 57% and 78%, and in PLS10 by up to 39% and 52%, with 25 and 50 μM of EA treatment, respectively (Figure 2B). Besides, the same concentration of EA also dramatically reduced the motility of the cancer cells. The inhibition rate in PC3 was about 41% and 77%, and in PLS10 by up to 13% and 67%, with 25 and 50 μM of EA, respectively (Figure 3A and 3B).

EA did not affect the secretion of MMP-2, MMP-9 from PC3 and PLS10 cells

Zymography showed that the secretion of MMP-9 from both PC3 and PLS10 cells were not reduced by EA

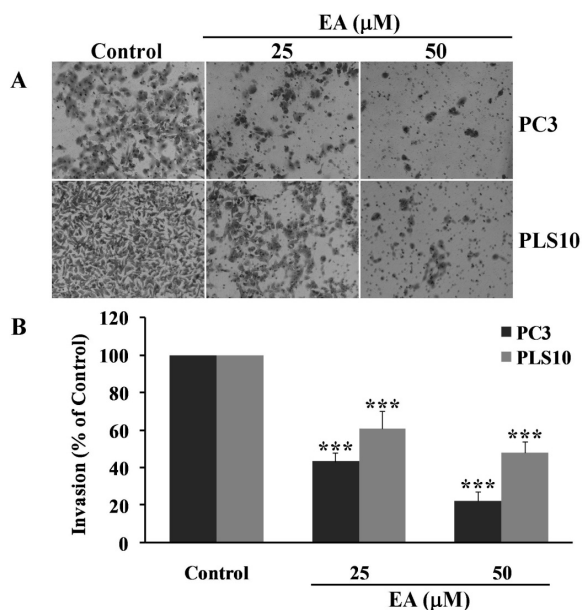


Figure 2. Effects of EA Treatment on Invasion of Prostate Cancer Cell Lines. The cells were photographed under phase-contrast microscopy (A) and quantified (B). The results are expressed as a percentage of the control (0 μM). ***significantly different from the control at $p < 0.001$

Meanwhile, MMP-2 (active-form) secretion from the cells was slightly reduced with 50 μM of EA treatment (Figure 4A and 4B). Collectively, these results indicated that EA did not inhibit the cancer cells invasion through the regulation of MMP-2 and MMP-9 secretion.

Effect of EA on the activity of collagenase/gellatinase

Several MMPs exhibit the activity of collagenase and/or gellatinase, the changing activity of collagenase/gellatinase in culture supernatant of PLS10 treated with EA were measured. Comparing to non-treated cells, the activity of collagenase/gellatinase was significantly decreased 24%, 36% and 49% in the culture supernatant of PLS10 treated with EA 25, 50 and 100 μM , respectively (Figure 5A). Moreover, the proteolytic activity of purified collagenase type IV was significantly

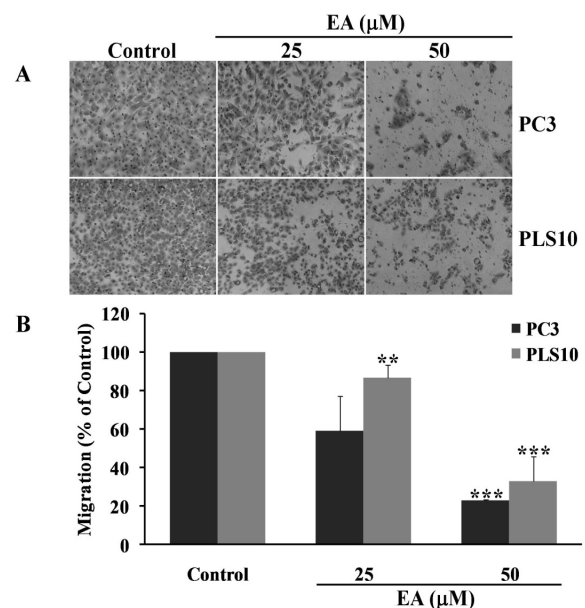


Figure 3. Effects of EA Treatment on Migration of Prostate Cancer Cell Lines. The cells were photographed under phase-contrast microscopy (A) and quantified (B). The results are expressed as a percentage of the control (0 μM). **, ***significantly different from the control at $p < 0.01$ and 0.001, respectively

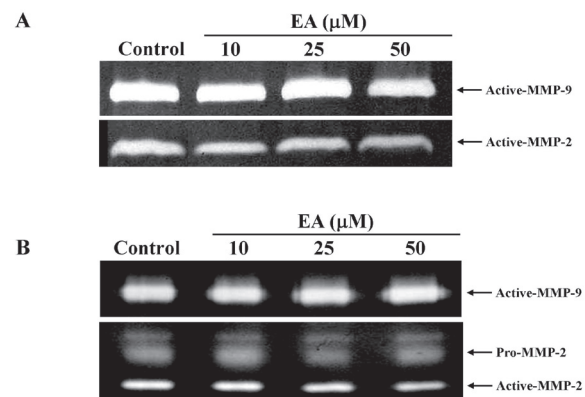


Figure 4. Effects of EA on Secretion of MMP-2 and MMP-9 from PC3 and PLS10 Cells. Secretion of MMP-2 (progenitor and active forms) and MMP-9 in the medium of PC3 (A) and PLS10 cells (B) is shown

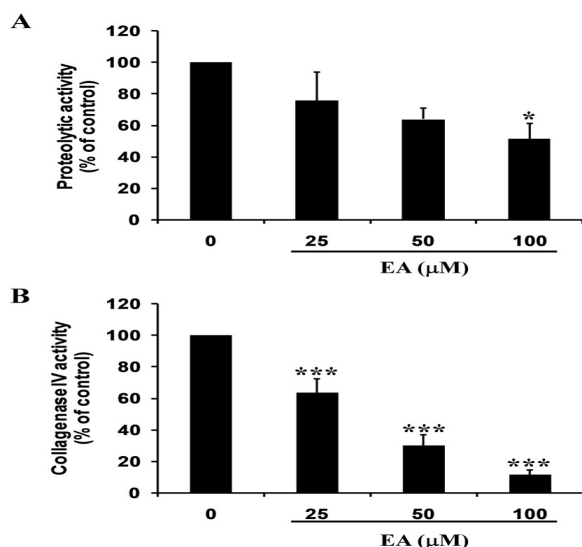


Figure 5. Effects of EA on Activity of Collagenase/Gelatinase Secreted from PLS10 cells (A) and Purified Collagenase Type IV from *Clostridium histolyticum* (B). The results are expressed as a percentage of the control (0 μM). *, ***significantly different from 0 μM at $p < 0.05$ and 0.001, respectively

inhibited by up to 32%, 68% and 87% with 25, 50 and 100 μM of EA, respectively (Figure 5B).

Discussion

EA has been shown to have a biological effect on prostate cancer cells both *in vitro* and *in vivo*. It has been shown to initiate cell cycle arrest, apoptosis and anti-tumorigenic activity in animal models (Castonguay, 1997; Longtin, 2003; Seeram et al., 2005; Bell and Hawthorne, 2008). It can also induce cell-cycle arrest and apoptosis in other cancer types, such as human cervical, bladder and leukemia cells maintained in culture (Narayanan et al., 1999; Li et al., 2005; Khanduja et al., 2006).

The important aspect of high incidence of morbidity and mortality in prostate cancer is tumor invasiveness and metastasis. Metastasis is a complex cascade, accompanied by various physiological alterations involved in extracellular matrix (ECM) degradation, which allows cancer cells to invade blood or lymphatic system spreading to another tissue or organ. Recently, it has been reported that EA inhibited the invasion of human androgen-independent prostate cancer, PC3 cells, *in vitro* (Lansky et al., 2005), however, the mechanism which related to the inhibition remains unclear. The present study, treatment with non-toxic concentration of EA on androgen-independent prostate cancer, PC3 and PLS10 cells markedly reduced the motility and the invasion of the cells. Previous study reported that cells-ECM interactions are premised to be essential for invasion, migration, and metastasis of tumors (Liotta et al., 1986; Azzam and Thompson, 1992; Gilles et al., 1997; Westermarck and Kahari, 1999). MMPs,

play a key role in ECM degradation for tumor growth, angiogenesis and invasion (Westermarck and Kahari, 1999). Among MMPs, MMP-2 and MMP-9 have been reported to be the most important for degradation of type IV collagen, a major component of basement membrane (Stetler-Stevenson, 1990; Giancotti and Ruoslahti, 1999; Zeng et al., 1999) and correlated with an aggressive, invasive or metastatic tumor phenotype (Bianco et al., 1998; Cockett et al., 1998; Papatoma et al., 2001; Wang et al., 2003). Therefore, MMP-2 and MMP-9 are afforded to be therapeutic targets of anticancer drugs. We found that EA treatment did not significantly affect the secretions of MMP-2 and MMP-9 from the cells by gelatin zymography. It revealed that the inhibition of EA on the cells invasion might not via the reduction of MMP-2 and MMP-9 secretions. Therefore, the inhibitory effect of EA on broad collagenase/gelatinase was determined. Interestingly, EA exhibited the inhibitor properties against the collagenase/gelatinase secreted from PLS10 cells and also the purified collagenase type IV.

Several articles revealed that MMPs are secreted by tumor cells themselves or by surrounding stromal cells stimulated by the nearby tumor. Numerous studies have linked altered MMP expression in different human cancers with poor prognosis. MMP-1, -2, -3, -7, -9, -13 and -14 all have elevated expression in primary and/or metastasis tumor, while up-regulation of MMPs lead to enhanced cancer cell invasion. From these results, we can conclude that EA had ability to reduce the invasiveness of prostate cancer cell lines by the modulation of MMP activity. Therefore, EA might be used as an adjuvant therapy for prostate cancer.

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