Evaluating the effects of ellagic acid on pSTAT3, pAKT, and pERK1/2 signaling pathways in prostate cancer PC3 cells

ABSTRACT

Objective: One of the most common malignancies among men is prostate cancer. Ellagic acid (EA), a polyphenol antioxidant, has many pharmacological actions, especially anticancer effects. The purpose of this study was to evaluate the effect of EA treatment on interleukin-6 (IL-6) gene expression, cell viability, IL-6 secretion, phosphorylated STAT3, ERK, and AKT cellular signaling proteins in human prostate cancer cells (PC3).

Materials and Methods: The cytotoxic effects of the EA (0-100 µM) on PC3 cells were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. IL-6 gene expression was down, using real-time quantitative polymerase chain reaction. The cellular concentration of phosphorylated ERK1/2, AKT, and STAT3 signaling pathways was assessed by Western blotting technic.

Results: EA treatment of PC3 cells resulted in a reduction of cell viability and phosphorylated STAT3, ERK, and AKT signaling proteins after 72 h in a dose-dependent manner. IL-6 gene expression and IL-6 levels significantly increased (P < 0.05) in a dose-dependent pattern in treated PC3 with EA. Thus, these data suggested the essential role of signaling proteins in EA-mediated anti-proliferation of PC3 cells.

Conclusions: Our finding shows that EA can be considered as a potent agent that decreases cell proliferation through a reduction of phosphorylated STAT3, ERK, and AKT cellular signaling proteins.

KEY WORDS: Antioxidant, ellagic acid, interleukin-6, PC3 cells, pomegranate

INTRODUCTION

Prostate cancer (PC) is one of the most common cancers in men. It is initially dependent on androgens for proliferation but eventually progresses to be androgen-independent phenotype.[1-3] PC in the androgen-independent phenotype becomes resistant to secondary endocrine therapy and chemotherapy.[2] Furthermore, PC in the form of the androgen-independent is accompanied by several molecular changes, especially the elevation of the serum interleukin-6 (IL-6). IL-6, a multifunctional cytokine, which is involved in the growth and differentiation of various types of malignant tumors, including breast, ovarian, lung, cervical, and prostate carcinomas.[15] There is increasing evidence suggesting that IL-6 is involved in the transition of PC from an androgen-dependent to an androgen-independent state.[2,4] IL-6, through its receptor, utilizes many cellular mediators and transducers such as Janus kinase-signal transducers and activators of transcription (JAK-STAT), phosphorylated ERK1/2 (T202/Y204), and phospho-AKT (S473).[6,7] The critical component of JAK-STAT pathway is pSTAT3. Therefore, the JAK-STAT pathway is considered as a possible target for novel therapies in oncology.[6,11]

In previous studies have demonstrated that a high intake of fruit and vegetables is associated with a reduced risk of common types of diseases and cancers.[6,9] Pomegranate (Punica granatum) contains flavonoid and polyphenol compounds.[10] Ellagic acid (EA) is the major polyphenol in pomegranate, raspberries, blackberries, strawberries, and nuts. EA has been reported to have anti-inflammatory, antioxidant, and anti-tumorigenic activity, and it is a promising anticancer agent.[8,9] This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

induces apoptosis in many types of cancers including breast, lung, colon, and PC.\textsuperscript{[11-13]} In previous studies have shown that EA not only induces apoptosis, but it has also been shown to initiate cell cycle arrest and modulating of the PI3K/AKT pathway.\textsuperscript{[10,14]} Therefore, considering the antioxidant properties of EA, this study was undertaken to evaluate the effects of EA on the IL-6 gene expression and phosphorylated cellular signaling proteins (pSTAT3, pAKT, and pERK1/2) in human PC3 cell line.

MATERIALS AND METHODS

The human PC3 cell line was obtained from the Pasteur Institute of Iran (Tehran, Iran). RPMI1640 medium, dimethyl sulfoxide (DMSO), trypan blue, and EA were obtained from Sigma (St. Louis, MO, USA). PEN/STREP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetus bovine serum were prepared from Gibco (Rockville, MD, USA). Antibodies were purchased from Abcam (San Francisco, CA, USA). All other chemicals used were of analytical grade.

Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

PC3 prostate cancer cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified 5% CO\textsubscript{2} incubator. The cells were seeded at 5000 cells/cm\textsuperscript{2} in 96-well plates and were allowed to attach for 24 h before treatment. After 24 h, media was removed and replaced with media containing different concentrations of EA (0, 10, 20, 30, 40, 50, 70, and 100 µM dissolved in DMSO with 0.1% final concentration) for 72 h. Each test was carried out 3 times. Then, the cytotoxic effects of the EA on PC3 cells were determined by MTT assay.\textsuperscript{[15]} Briefly, after treating cells for 72 h, the medium of each well was removed and was replaced with 100 µL colorless RPMI and 10 µL MTT (5 mg/mL in phosphate buffered saline) and incubated for 4 h. Then, the medium was removed, and formazan was solubilized in 150 µL DMSO. Formazan absorbance was measured at 490 nm using a microplate reader (Stat Fax 3200, Awareness Technology, USA). The viability percentage was calculated as follows: Percentage viability = A (sample)/A (control) × 100.

Real-time quantitative polymerase chain reaction for interleukin-6 expression

PC3 prostate cancer cells were harvested after treatment with different concentrations of EA (0, 30, 50, and 70 µM) in 6-well plates for 72 h. The total mRNA of the cells was extracted using Biozol reagent according to the manufacturer's instructions. The total mRNA concentration and quality were measured by 260/280 nm absorbance ratio using NanoDrop spectrophotometer (Thermo-USA). cDNA prepared from RNA using a synthesis kit (Takara Bio Inc., Japan) and utilizing 1 µg total RNA according to the manufacturer's instructions. The procedure of cDNA reverse transcription was carried out using Prime Script\textsuperscript{™} reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. Later, cDNA was amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using SYBR\textsuperscript{®} Green PCR Master Mix (Qiagen, Germany) in the presence of specific primers for IL-6 (Forward: 5'-AACCCAGACTGTCAGTGATA-3'; Reverse: 5'-TGTCCTGACGCCACTGTT-3') and GAPDH (Forward: 5'-ACACCACCTCCTCACTTTG-3'; Reverse: 5'-CCACACCTGTGCTGTA-3'). The primers were designed using Oligo 6.0 (Molecular Biology Insights, Cascade, CO, USA) and confirmed by the blast (NCBI). They were purchased from Eurogentec (Seraing, Belgium). IL-6 gene expression was detected using Rotor-Gene 3000 (Corbett, Australia) for each mentioned concentration.\textsuperscript{[16]} The temperature profile for the reaction was an initial denaturation stage of 95°C at 10 min, then a three-step program was developed for 40 cycles including 95°C for 15 s, 60°C for 20 s, and 72°C for 25 s, respectively. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene was used as an endogenous control gene for the normalization of IL-6 expression.

Western blotting

PC3 cells were grown in RPMI 1640 medium with different concentrations of EA (0, 30, 50, and 70 µM), and they were harvested after 72 h. Then, the cells were lysed on ice using RIPA buffer (50 mM Tris-HCl with pH 7.5, 150 mM NaCl, 1% v/v Triton 100X, 0.5% v/v sodium deoxycholate, 1 mM EDTA, 0.1% v/v sodium azide, 50 mM NaF, 0.1% sodium dodecyl sulfate [SDS], and 1 mM PMSF).\textsuperscript{[15]} Protein concentrations for Western blot analyses were measured by NanoDrop-2000 Spectrophotometer. The protein lysates were mixed with an equal volume of loading buffer (0.125 M Tris-HCl with pH 6.8, 4% of SDS, 20% of glycerine, and 10% of 2-mercaptoethanol) and then a three-step program was developed for 40 cycles including 95°C for 15 s, 60°C for 20 s, and 72°C for 25 s, respectively. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene was used as an endogenous control gene for the normalization of IL-6 expression.

Membranes placed into a blocking solution (5% w/v skim milk powder in TBS-Tween buffer (containing 10 mM Tris pH 7.4, 100 mM NaCl, and 0.1 mM Tween-20) for overnight at 4°C.\textsuperscript{[17]} Later, the membranes were washed 3 times in TBS-Tween buffer for 10 min, and they were incubated with primary antibodies against either phosphorylated STAT3, phosphorylated p44/42 ERK1/2, phosphorylated AKT, or β-actin according to the manufacturer’s protocols at room temperature for 3 h. The membranes were washed with TBS-Tween buffer 3 times for 10 min, and they were incubated with an appropriate dilution of horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. After washing the membrane 3 times for 10 min in TBS-Tween buffer, the bands were revealed by adding BM Blue POD substrate.
Measurement of IL-6
PC3 cells were cultured and they were treated with different concentrations of EA (0, 30, 50, and 70 µM) at 37°C in a 5% CO₂ incubator in 6-well plates for 72 h. The levels of IL-6 in the supernatants were assessed using an ELISA kit (AViBion Human IL-6 ELISA kit) according to the manufacturer’s protocols. Optical density was recorded by an ELISA plate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology, USA).

Statistical analysis
Data were shown as mean ± standard deviation. Statistical analysis was performed using SPSS version 20.0 software (SPSS, Chicago, IL, USA). Inhibitory concentration of 50% (IC₅₀) value was calculated from dose response curve for three individual values. For expression analysis, the relative levels of quantitative gene expression were estimated with the 2^{−ΔΔCT} method and the data were normalized for GAPDH and expressed as fold change. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. Western blot experiments were repeated 3 times. The values of P < 0.05 were considered to be statistically significant.

RESULTS

Inhibition of cell proliferation by ellagic acid
Figure 1 shows the treated PC3 cells with EA decreased cell proliferation and resulted in morphological changes after 72 h in a dose-dependent manner. PC3 cells that exposed to EA (0-100 µM) exhibited an IC₅₀ about 50 µM [Figure 2].

The effect of ellagic acid on interleukin-6 expression in PC3 cells
Figure 3 shows the treated PC3 cells with/without EA for investigating IL-6 expression using RT-qPCR. IL-6 expression in treated PC3 with EA significantly upregulated (P < 0.05) in a dose-dependent pattern. As it is shown in Figure 3, there was a significant elevation (P < 0.05) in IL-6 expression by almost 3.2, 14.7, and 18.2 fold at 30, 50, and 70 µM concentrations, respectively, in EA-treated PC-3 cells when compared with the control cells.

Effects of ellagic acid on the cellular signaling pathways in PC3 cells
Figure 4 shows the effect of EA on the cellular levels of phosphorylated STAT3, ERK, and AKT signaling proteins. Our Western blot data indicated a decrease in the cellular concentrations of pSTAT3, pAKT, and pERK1/2 signaling pathways in a dose-dependent manner, when compared with the control cells after treatment with different doses of EA [Figure 4] for 72 h.

Effect of ellagic acid on interleukin-6 secretion
IL-6 production by cultured PC3 cells is shown in Figure 5. The treatment of PC3 cells with different concentrations of EA led to the elevation of IL-6 in culture supernatants in a dose-dependent manner. There was a noticeable increase (P < 0.05) in IL-6 levels (119.3%, 256.6%, and 365.9% at 30, 50, and 70 µM of EA, respectively) in EA-treated PC-3 cells as opposed to the control cells.

DISCUSSION
Nowadays, cancers are considered as dangerous diseases and a major health hazard for humans in the world. In previous studies have shown that EA have many biological effects on PC cells both in vitro and in vivo such as cell cycle arrest, apoptosis, and anti-tumorigenic activity in animal models.[16] In our study, EA resulted in a reduction of PC3 cells viability [Figure 2]. Furthermore, in this study EA, a polyphenol compound has shown a strong potential anti-proliferative effects on PC3 cells [Figure 1] as reported by other investigators.[17,18] EA was considered as a promising new therapeutic anticancer agent, with both anti-proliferative and pro-differentiating properties,[19] which is in agreement with our study.
Nevertheless, in our study EA resulted in an elevation of IL-6 secretion by PC3 cells [Figure 5]. Many previously published studies have reported that IL-6 can be inhibited or stimulated in cancer cell lines due to its different interactions with cellular regulatory signaling pathways. Therefore, the elevation of IL-6 can be resulted from an internal reaction of PC-3 cells against anti-proliferative effects of EA because IL-6 leads to the increasing of cancer cell resistance against chemotherapeutic agents. In this study, RT-qPCR confirms the elevation of IL-6 expression due to the effect of EA [Figure 3]. Several studies demonstrated that the administration of EA resulted in the elevation of other cytokines such as IL-10 which is consistent with our study. Nevertheless, it is reported that EA has anti-inflammatory properties due to its effects on the isoform of NO synthase, cyclooxygenase-2, tumor necrosis factor alpha, and IL-6 down-regulation through the inhibition of nuclear factor-kappa B. Also, it is reported that EA can lead to a significant increase in p21 expression, a negative regulator of the cell cycle. Therefore, it seems that EA has different effects on gene expression in tumors cell lines. In our study, in spite of the elevation of IL-6 expression, EA had shown a strong potential anti-proliferative effect on PC3 cells [Figure 1]. In previous studies have shown that EA induces apoptosis through reducing the PI3K/AKT, JAK-STAT, and ERK1/2 MAPKs pathways, which is in agreement with our study.

In a reported study, it was demonstrated that EA induces apoptosis and subsequently suppresses colon cancer through the PI3K-AKT signaling pathway. Furthermore, EA causes a remarkable decrease in Bcl-2 levels, whereas pro-apoptotic protein Bax level was increased. In our results, cellular AKT signaling protein had diminished by EA which can be a reason for reducing cell proliferation. On the other hand, many published papers suggested that EA inhibits PI3K/AKT signaling by modulating the apoptotic molecules such as Bcl-2, Bax, and caspase-3. Thus, another possible mechanism for anti-proliferative effects of EA on PC3 cells, at least in part, can be applied by inhibiting PI3K/AKT signaling pathway. As well, our findings demonstrated that JAK-STAT signaling pathway reduced by EA and it can be considered an additional reason for reducing viability in PC3 cells. Published studies showed that STAT3 protein prevents apoptosis and contributes to cell survival by increasing of anti-apoptotic factors such as Bcl-2.
as Bcl-2 and Bcl-XL. Therefore, in this study, the reduction of STAT by EA cell, at least in part, led to decline PC3 cells survival in spite of the elevation of high IL-6 expression. On the other hand, in this study EA caused a reduction in ERK1/2 protein signaling in PC3 cells, which is in line with other studies that indicate EA diminished the activation of the ERK1/2 pathway [Figure 4]. Many reports suggest that the activation of MAPK/ERK1/2 can be associated with cell survival. Therefore, inhibition of MAPK/ERK1/2 causes a reduction in cell survival as seen in this study. Likewise, recent findings suggest that MEK-1/MAPK (ERK1/2) pathway may play a role in suppressing apoptosis due to its interface with survival signaling at the level of Bcl-2 to directly link these two critical growth pathways. On the other hand, previous studies have shown that EA can reduce the activation of p38, JNK, and MAPKs/ERK1/2 preventing the inhibitory protein IkB-degradation and inducing an inhibition of the nuclear translocation level of p65 in colonic mucosa. Also, MAPK has a key role in inducing gene expression due to its transmitting of extracellular inflammatory signals into intracellular responses. EA, however, potently inhibits the activation of MAPKs. Thus, the reduction of ERK1/2 by EA in this study, at least in part, resulted in diminishing of PC3 cells survival, in spite of the elevation of high IL-6 expression.

In this study, we did not evaluate the effect of EA on other protein signaling pathways such as the nuclear translocation level of p65, caspase-3, and regulatory effectors such as Bcl-2 levels and Bax levels. These factors can influence cell apoptosis and survival. Therefore, we suggest that future studies focus on the effects of EA on other cell signaling and effector proteins.

CONCLUSIONS

Our finding indicates that EA can be considered as a potent agent that decreases PC3 cell proliferation through the reduction of ERK1/2, AKT, and STAT3 cellular signaling proteins.

Acknowledgments

We would like to express our gratitude to those who have helped us in Clinical Biochemistry Research Center of Shahrekord University of Medical Sciences.

Financial support and sponsorship

This study was funded by Shahrekord University of Medical Sciences (grant no. 1144), Shahrekord, Iran.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

5. Taniguchi K, Karin M. IL-6 and related cytokines as the critical lymphins between inflammation and cancer. Semin Immunol 2014;26:54-74.


