Ellagic Acid Exerts Apoptotic and Antiangiogenic Activity on Human Intestinal Cancer (Caco-2) Cell Line

Mehmet Ali Kisacam¹

¹Department of Biochemistry, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, (31060) Hatay/ Turkey

ORCID; 0000-0003-0521-9434

Abstract: Colon cancer is a global health issue that affects increased rates of people every year. Angiogenesis and escape from apoptosis are important features of colon cancer. Ellagic acid is a bioactive polyphenol that might show antiangiogenic activity and induce apoptosis. Despite the potential effects of EA, there is a lack of knowledge of how it exerts its effects. In this study, we focused on determining the effects of ellagic acid on biological markers, taking part in colon cancer development. The effects of EA were determined through the evaluation of Fyn and Hck gene expression levels together with Bcl-2, Bax, VEGF, and HO-1 protein levels. Moreover, cytotoxic effects of EA were evaluated with the MTT test. IC50 values of EA on Caco-2 cells were 80.89 uM, 24.67 uM, 3.67 uM at 24, 48th, and 72nd hours, respectively. EA application (10, 20, 40, and 100 uM) decreased Bcl-2 levels significantly, while it increased Bax protein levels compared to control (p<0.001). EA exposure at 100 uM concentration decreased both HO-1 and MMP-7 levels significantly. VEGF protein levels were found to decrease at 40 and 100 uM concentrations of EA. Fyn gene expression levels remained unchanged among control, 10 uM and 20 uM EA concentration while it decreases significantly at 40 and 100 uM EA concentration. Hck gene expression decreased significantly at 20, 40, and 100uM EA concentrations. In conclusion, EA exhibited a protective role against colon cancer development both by inducing apoptosis via inhibition of Fyn, Hck, HO-1 and also by inhibiting angiogenesis.

INTRODUCTION

Colon cancer is one of the commonly seen cancer that cause increased rates of morbidity and mortality worldwide ¹. Almost 1.4 million new patients were diagnosed with colon cancer and more than 500,000 people died globally in 2012. New patients diagnosed with colon cancer are estimated to increase 60% and mortality rates to 1.1 million by 2030 ².

New blood vessel formation from the old ones is described as angiogenesis ³. Oxygen diffusion from vessels is vital for tissues and oxygen deficiency leads to hypoxia-related oxidative stress which is ended with apoptosis ⁴. Heme oxygenase (HO-1) which catalyzes heme to biliverdin is reported to protect tumor cells from hypoxic stress and apoptosis ⁵. HO-1 overexpression has been showed to protect colon cancer cells from oxidative stress, apoptosis and to cause cell proliferation ⁶,⁷. Cancer cells have atypical metabolic features to guarantee survival cancer cells escape from apoptosis or to form new blood vessels ⁸,⁹. Vascular endothelial growth factor (VEGF) is a vital factor modulating angiogenesis which participate in initiation and progression of colon cancer ¹⁰. VEGF also might act as apoptosis inhibitor ¹¹. Matrix metalloproteases (MMP) play a key role in angiogenesis by breaking down the extracellular matrix and liberating VEGF ¹¹,¹². Unlike other MMPs, MMP-7 is mainly produced in cancer cells and increased levels of MMP-7 have been demonstrated in colorectal cancer ¹¹,¹². MMP-7 increases cancer cell metastasis and targeting MMP-7 has been shown to have beneficial effects in cancer treatment. Therefore, targeting MMP-7 and managing angiogenesis at early stage could give the opportunity to prevent metastasis or progression of cancer ¹¹. In addition to angiogenesis ongoing reactive oxygen species exposure might cause pathological conditions such as DNA damage and mutations that may lead to colon cancer development. Cells induce apoptotic cell death under critical conditions occur in mitochondria such as deteriorated membrane permeability, impaired inner membrane integrity, and release of cytochrome c ¹₄,¹₅. Apoptosis together with metabolic changes take part in colon cancer formation ¹₆. Bcl-2 and Bax proteins, which play role in the regulation of apoptosis, are regarded respectively as anti-apoptotic and pro-apoptotic proteins ¹₇. Therefore, the determination of Bcl-2 and Bax levels might be subsidiary to evaluate the role of apoptosis in colon cancer. As much as angiogenesis, controlling oxidative stress together with apoptosis could be beneficial in reducing colon cancer development ⁸,¹⁹.

Src kinases such as Fyn and the hematopoietic cell kinase (Hck) have been indicated to modulate cell proliferation and apoptosis ²⁰. Overexpression of these kinases has been reported in several epitheli-
al cancers such as colon cancer. Increased levels of Src kinases also indicated to be related with malignity in cancers. Fyn inhibition has been indicated to result with decreased cell death, while its activation has been reported to exhibit anti-apoptotic effects by inhibiting caspase cleavage in Hela cells. Similarly, Hck knockdown has been reported to induce apoptosis via increasing Bax levels.

Reducing or incapacitating cancer with promising compounds has been investigated currently by scientists. Polyphenols are the promising compounds due to their potential bioactive capacities. Ellagic acid is a compound that contains phenolic groups, together with lactones connected with hydrogen bonds which give the electron acceptor feature to the molecule. Woody plants, berries, grapes and pomegranates are important source of ellagic acid. Biologically ellagic acid is a bioactive polyphenol that show antiproliferative activity and induce apoptosis in many human cancer cell lines. Additionally, the potential of ellagic acid to show antiproliferative activity was attributed to the interaction capacity with cell membrane proteins and cover their binding sides. Despite the information about ellagic acid, still there is a lack of knowledge about how ellagic acid acts at the cellular and molecular levels. In this study, we focused on determining the interaction of biological markers, taking part in colon cancer development, and the effects of EA on these markers.

**MATERIALS and METHODS**

**Chemicals**

Human colon cancer (Caco-2) cells were obtained from the American Type Culture Collection (ATCC). Cell culture mediums were purchased from Gibco. EA was purchased from Alfa Aesar, Germany (A15722 Lot: 10176718, Ellagic acid hydrate). Cell Proliferation Kit I (MTT, cat. 11 465 007 001) was purchased from Roche. Protease-phosphatase inhibitor cocktails (cat. 88666 and 78420), RIPA buffer (cat. 89900), Pierce™ BCA Protein Assay Kit (cat. 23227), anti-Bcl-2 (cat. MA5-11757), anti-Bax (cat. MA5-14003) and anti-VEGF (cat. MA5-15157) antibodies together with anti-mouse horse radish peroxidase conjugated secondary antibody (cat. 32430) and anti-rabbit horse radish peroxidase conjugated secondary antibody (cat. 32460) were obtained from Thermo Scientific. Anti-HO-1 (TA348005) and Anti-MMP-7 (TA349925) were obtained from Origene, Herford, Germany. GAPDH (MA5-15738) were purchased from Thermo Scientific. PureLink™ RNA Mini Kit (cat. 12183018A) and High-Capacity cDNA Reverse Transcription Kit (cat. 4374966) were obtained from Thermo Scientific. Hck, Fyn and GAPDH TaqMan Gene Expression Assay (Hs00176628_m1, Hs01067403_m1 and Hs02786624_g1) and Taqman Universal Master mix (cat. 4440038) were purchased from Thermo Scientific (USA).

**Cell viability assay**

MTT assay was used to determine the effects of EA on the proliferation of cultured cancer cells. Briefly, 1x10^3 cells/well were passed into of 96 multi-well plates and maintained at 37°C under 5% CO2 humidified conditions for 24 h in DMEM (containing 10% fetal bovine, 1% L-glutamine and 1% penicillin-streptomycin). After cells becoming monolayer, culture media was aspirated, increased concentrations of EA (0.2-100 µg/ml) exposed to Caco-2 cells for 24, 48 and 72 h. Subsequently, the medium was removed and cells were incubated for 4 h in MTT reagent. After 4 h, the medium was aspirated, and formazan was dissolved in 100 µl solubilization reagent. The plates were incubated overnight at 37°C under 5% CO2 humidified conditions and the absorbance was read at 570 nm with the plate reader (MultiscanGo; Thermo). Concentration that inhibited 50% of the cell growth (IC50) was determined via GraphPad Prism 6.0 program.

**Estimation of apoptotic and angiogenetic effects of Ellagic acid**

Effects of EA on Bcl-2, Bax, VEGF, MMP-7 and HO-1 protein levels of Caco-2 cells after 48 hours incubation were determined by Western Blot analysis. Briefly, Caco-2 cells were cultured in 6 well plates at 1x105 cells/ml density. Following cell attachment, they were treated with the increasing concentrations of EA. Cells were harvested in ice cold PBS and centrifuged. The cells resuspended in RIPA lysis buffer containing protease inhibitor cocktail. The protein concentration was determined with Pierce™ BCA Protein Assay Kit. Equivalent quantities of extracted proteins were separated on a 10% SDS/PAGE gel. Subsequently proteins were transferred onto nitrocellulose membrane. Membranes were incubated in BSA and then blocked for 1 h. Following blocking, the membranes were left overnight in the primary antibody at 4°C. After adequate washing with PBS-T, membranes were incubated with the secondary antibody. Protein bands were visualized by using the ECL chemiluminescence detection kit.

**Determination of the mRNA expression level of SFKs members**

The gene expression levels of Hck and Fyn were measured by quantitative polymer chain reaction (qPCR) analysis. Briefly, total RNA was isolated using PureLink™ RNA Mini Kit according to the manufacturer's instructions. The concentration and purity of RNA samples were measured spectrophotometrically. High-Capacity cDNA reverse transcription kit was used cDNA syntheses from RNA samples. qPCR analysis was performed with Taqman Universal Master mix and Taqman assays. GAPDH gene expression was used as internal control. Relative gene expression levels were calculated according to the 2-ΔΔCt method as described by Livak et al.

**Statistical Analysis**

The data presented as mean ± standard error (SE) for continuous variable. The cell viability among concentration and time groups was evaluated with two-way analysis of variance. When any statistical significant interaction term (concentration x time) were found, Simple Mean Effect Analysis was performed. Kruskal Wallis test was done to indicate the differences among the concentration groups in Western Blot analysis. In case of any statistical difference, Mann-Whitney U test with Bonferroni correction was used as post-hoc tests. Values less than 0.05 were considered to be statistically significant. All statistical analyses were performed by using IBM SPSS Statistics for Windows, Version 22.0.

**RESULTS**

**Cytotoxic effects of EA in Caco-2 cells**

The survival rates of Caco-2 cells were determined with MTT cell viability assay. A Two Way Anowa was performed to determine the effects of different concentrations of EA on Caco-2 cells (Table 1). Time and concentration were found to effect cell viability significantly (p<0.001). These results showed that EA application for 24 h, 48 h and 72 h groups affected Caco-2 cell viability in different manners and differed from each other. The highest cell viability was found at 24 h group and the cell viability observed to decrease gradually at 48 h and 72 h groups. Concentration dependent decrease of cell viability detected in 24 h, 48 h and 72 h groups. Moreover, there was a statistically significant interaction between time and concentration (p<0.001).

Simple main effect analyses showed that EA decreased cell viability significantly at doses greater than 5 µM, 2 µM, and 1 µM concentration at 24 h, 48 h, and 72 h, respectively. Ignoring the concentration effect, it was found that EA exposure decreased cell viability in different times. In general, as the dose increased, cell viability tended to decrease. Cell viability was determined to decrease as time increased at doses greater than 5 µM. IC50 values were found 80.89 µM, 24.67 µM, 3.67 µM at 24, 48th and 72nd, respectively. Inhibition half of the cells at 24.67 µM concentration together with the findings indicating lethal dose 50 values almost 20 µM at 48th hours makes us determine the time as 48th hours for further analysis (Table 1).

**Effect of EA on Bcl-2, Bax, VEGF, HO-1 and MMP-7 protein levels**
In a study evaluating in vitro antiproliferative effects of EA, IC50 values of EA were found to be 37 uM for Caco-2 cells for 48 hours incubation in EA containing culture conditions. Effects of EA on Bcl-2, Fyn, and Hck gene expression of Caco-2 cells after 48 hours incubation in EA containing culture conditions. EA application (10, 20, 40, and 100 uM) decreased Bcl-2 levels significantly (p<0.001) compared to control. EA treatment at different concentrations was comparable among the groups (10, 20, and 40 uM), yet 100 uM EA application decreased Bcl-2 levels significantly (p<0.001) compared to control concentrations (10, 20, and 40 uM). Hek gene expression levels were comparable at control and 10 uM EA concentration compared to control (Figure 1A). EA treatment at different amounts of EA (10, 20, 40, and 100 uM) showed a significant increase in Bax protein expression levels compared to control (p<0.001, Figure 1B). The highest Bax protein values were determined in 10 uM EA concentration compared to the other EA concentrations (20, 40, and 100 uM). Apart from 40 uM EA exposure, HO-1 protein levels decreased significantly compared to control in Caco-2 cells (Figure 2A). VEGF protein levels were found to increase significantly in 10 and 20 uM EA concentration while it decreased at 40 and 100 uM concentration of EA (Figure 2B). Although MMP-7 protein levels increased following 10, 20 and 40 uM EA exposure, 100 uM EA exposure decreased MMP-7 protein levels significantly (Figure 2C).

Effects of EA on Fyn, and Hck gene expression levels

Fyn gene expression levels remained unchanged among control, 10 uM and 20 uM EA concentration while it decreases significantly at 40 and 100 uM EA concentration compared to control (Figure 3A). Hck gene expression levels were comparable at control and 10 uM EA concentration (Figure 3B). Moreover, compared to control statistically significant downregulation was found in 20, 40 and 100 uM EA concentrations (Figure 3B).

Discussion

In a study evaluating in vitro antiproliferative effects of EA, IC50 values of EA were found to be 37 uM for Caco-2 cells for 24 hours incubation. In their study they also have reported that Caco-2 cells were the most sensible cell line compared to DU145, Hs578T and MCF-7 cell lines. Carota et al. reported that EA exposure for 48 hours killed 40 or 50% of DU145 cells while administered at 50 and 100 uM concentrations, respectively. Kumar et al. demonstrated that HeLa cells exposed to EA for 72 hours dose dependently died and IC50 dose are reported to be 19.47 uM. Correlated to these studies in our study EA showed showed relatively identical IC50 dose in Caco-2 cells.

Intestine, due to its feature, faces reactive oxygen radicals continuously. Continuous ROS exposure was indicated to be responsible for many disorders such as colon cancer. Increased ROS formation causes increased levels of HO-1 in Caco-2 cells. Ellagic acid, on the other hand, is reported to decrease HO-1 levels in DU-145 prostate cells. Attia et al. reported that EA can be used to reduce the effects of colon cancer due to its beneficial effects. In another study...
conducted in prostate cells EA decreased HO-1 levels 23. In our study except from 40 uM, EA exposure to Caco-2 cells resulted in decreased HO-1 levels. Our findings showed that EA might decrease Caco-2 cell proliferation at 100 uM concentration. MMP-7 is mainly produced in cancer cells and increased levels of MMP-7 have been demonstrated in colorectal cancer. MMP-7 increases cancer cell metastasis and participates in angiogenesis by setting VEGF free. Ellagic acid derived-metabolites have been found to decrease MMP-7 levels in prostate cancer cells 35. Interestingly, in our study, EA decreased MMP-7 protein levels at a 100 uM dose, concluding that EA shows an inhibitory effect on Caco-2 cells when administered more than IC50 doses. Several studies indicated that HO-1 directly and indirectly (via VEGF) affects angiogenesis 34,36. Furthermore, angiogenesis is reported to be linked with tumor progression and metastasis. Evaluation of angiogenesis is reported to accomplish with an important regulatory factor called VEGF 12. Losso et al. 13 found that EA application decrease Caco-2 cell proliferation via inhibition of VEGF. Similarly, Vanella et al. 34 reported that at 25-50 uM concentration of EA significantly decrease VEGF levels in LnCaP prostate cancer cells. Correlated to these studies, 40 and 100 uM EA exposure decreased VEGF levels in our study. Our results also in parallel with the evaluation that indicate HO-1, MMP-7 and VEGF levels are together take part in cancer cell proliferation via inducing angiogenesis 36; while EA application at doses higher than 40uM decrease HO-1, MMP-7 and VEGF levels. In addition to its role in angiogenesis, HO-1 has been reported to take part in the regulation of apoptosis in Caco-2 cells 6. In a study evaluating the effects of Banxia Xiexin decoction in colon cancer induced nude mice, it has been indicated that both HO-1 and Bcl-2 protein levels decreased following Banxia Xiexin decoction application, which is concluded as induction of apoptosis 37, Yousef et al. 38 reported increased mitochondrial Bax protein levels in Caco-2 cells exposed to EA and consequent apoptosis. Moreover, EA has been indicated to decrease Bcl-XL protein levels, which is a member of Bcl-2 family, and to cause apoptosis in Caco-2 cells 39. Overexpression of Src kinases have been reported in several epithelial cancers such as colon cancer. Elevated levels of these kinases especially attributed to malignity in cancers 21. Furthermore, Fyn inhibition has been indicated to be related with reduction in cell growth 22. On the other hand, activation of Fyn has been reported to exhibit anti-apoptotic effects by inhibiting caspase cleavage in Hela cells 23. Similarly, Hck knockdown has been reported to induce apoptosis via increasing Bax levels 24. Opposite to the Src kinases’ roles in cancer development, EA has been reported to have the potential to prevent tumor development by inhibiting invasion, angiogenesis, and cell death escaping capacity 1. In our study EA exposure decreased both Hck and Fyn levels which might be attributed to the antitumor potential of the EA.

Conclusion
In conclusion, our results indicated that EA exhibits a protective role against colon cancer development by inducing apoptosis via inhibition of Fyn and Hck. Besides, EA inhibits angiogenesis by reducing VEGF levels which are regarded as an important regulatory factor for the evaluation of angiogenesis. Furthermore, decreased HO-1 levels in our study might indicate the potential of EA to inhibit cancer cell escape from apoptosis and oxidative stress.

Conflict of interest
The authors declare that they have no conflict of interest.

Ethics Approval
The study is an in-vitro study, does not include human or animal samples, and thus do not need any ethical approval.

REFERENCES


