

The Effects on Biomarkers of Caffeic Acid Phenethyl Ester Applied to a Gastric Cancer Cell Line

Gastrik Kanser Hücre Hattına Uygulanan Kafeik Asit Fenetil Ester'in Biyomarkerleri Üzerine Etkileri

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ABSTRACT

Gastric cancer has a high incidence and is one of the leading causes of mortality both in males and females. Angiogenesis is the formation of new capillaries from existing blood vessels through which oxygen and nutrients are transported to the tissues to promote growth, and it plays a pivotal role in the invasion and metastasis of cancer caused by oncogenes. Even though this process is useful in tissue repair and normal growth, it also facilitates the growth of cancer tissues and their metastasis via the hematogenous route, and in this way, it speeds up metastasis and invasion. In the present study, we aimed to investigate the relationship between angiogenesis and markers such as VEGF, MMP, TSP-1 and Endostatin in a CAPE-administered gastric cancer cell in culture. Following CAPE administration, cytotoxicity was measured by MTT test. Angiogenic behavior of cells was examined immunohistochemically by VEGF, MMP and Endostatin, and TSP – 1 level was measured by RT-PCR test. MTT test showed that CAPE administered at a concentration of 0.5 µg/mL exerted a cytotoxic effect on a gastric cancer cell line. Gene expression depicted by RT-PCR showed that VEGF, MMP and TSP levels were decreased, while the endostatin level was increased. Changes in MMP, VEGF, TSP and endostatin suggest that CAPE has a significant therapeutic effect on gastric cancer. In the light of these findings, CAPE, which is a natural and inexpensive substance, could be effective in the treatment of cancer patients and advanced studies need to be carried out.

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ÖZ

Mide kanseri yüksek insidansa sahiptir ve hem erkeklerde hem de kadınlarda mortalitenin önde gelen nedenlerinden biridir. Anjiyogenez, büyümeyi desteklemek için oksijen ve besin maddelerinin dokulara taşındığı mevcut kan damarlarından yeni kılcal damarların oluşumudur ve onkogenlerin neden olduğu kanserin invazyonunda ve metastazında önemli bir rol oynar. Bu işlem doku onarımı ve normal büyüme için yararlı olsa da, kanser dokularının büyümesini ve hematojen yol yoluyla metastazını kolaylaştırır ve bu şekilde metastazı ve invazyonu artırır. Bu çalışmada mide kanseri hücre kültürüne uygulanan CAPE'nin VEGF, MMP, TSP-1 ve Endostatin gibi anjiyojenik ve anti-anjiyojenik belirteçler arasındaki ilişkiyi araştırmayı amaçladık. CAPE uygulamasını takiben sitotoksite, MTT testi ile ölçüldü. Hücrelerin anjiyojenik davranışı immünohistokimyasal olarak incelendi ve VEGF, MMP ve Endostatin ile TSP-1 seviyesi LC PCR testi ile ölçüldü. MTT testi, 0,5 µg/mL'lik bir konsantrasyonda uygulanan CAPE'nin, bir mide kanseri hücre hattı üzerinde sitotoksik bir etki yaptığını gösterdi. LC PCR ile gösterilen gen ekspresyonu, VEGF, MMP ve TSP seviyelerinin azalırken endostatin seviyesinin arttığını gösterdi. MMP, VEGF, TSP ve endostatindeki değişiklikler, CAPE'nin mide kanseri üzerinde önemli bir terapötik etkiye sahip olduğunu düşündürmektedir. Bu bulgular ışığında, doğal ve ucuz bir madde olan CAPE, kanser hastalarının tedavisinde etkili olabilir. Daha ileri çalışmaların yapılması gerekir.

Anahtar Kelimeler: Anjiogenezis, Hücre Kültürü, VEGF, MMP, CAPE, Endostatin, TSP-1.

INTRODUCTION

Cancer is an uncontrolled proliferation of cells and stands at the forefront of the health problems of our age. Gastric cancer is the second most common form of cancer in females and the third most common form of cancer in males worldwide. Cancer occurs when the genes undergo mutation and acquire cancer properties. These genes are referred

to as oncogenes (1). They exhibit tumorigenic activity and control cell proliferation, invasion and angiogenesis (2). Invasion by cancer cells is via blood vessels or the lymphatic system and this is facilitated by angiogenesis (3), which is defined as the formation of new capillaries from existing ones, providing oxygen and nutrients and removing waste. Angiogenesis is a fundamental factor in growth and differentiation and plays a critical role in tumor spread and metastasis (3,4,5). Many factors have been implicated in the development and metastasis of cancer. One of the most important activators of the angiogenic system is vascular endothelial growth factor (VEGF), a member of a group of growth factors (6). Angiogenic processes are regulated by growth factors including VEGF, simple fibroblast growth factor and hepatocyte growth factor (6). The presence of VEGF has been shown in many types of cancers including colon cancer, and its increase has been attributed to malignancy (7).

VEGF increases vascular permeability, stimulates matrix metalloproteinase (MMP), which is responsible for the disruption of the extracellular matrix and therefore facilitates metastasis and invasion (8). There is abundant evidence of angiogenesis being involved in cancer in pre-clinical and clinical settings. For example, increased intratumoral microvascular density and VEGF expression correlate with shorter relapse-free intervals and overall survival (9). One other angiogenic agent is MMP. Endothelial cells stimulated by VEGF initially synthesize MMPs. These MMPs then become free and disrupt the structure outside the blood vessels. This disruption expedites angiogenesis (7).

Endostatin is 20kDa fragment of collagen XVIII (10). As an early-identified endogenous inhibitor of angiogenesis with strong potential, endostatin was rapidly moved to clinical trials; however poor solubility and difficulty purifying the molecule through recombinant expression made it inappropriate for the clinic (9) it is a potent inhibitor of angiogenesis, directly preventing endothelial cell growth and migration and inhibiting the angiogenic effects of VEGF (11,12,13). Thrombospondin-1 (TSP-1) is a relatively large, heparin-binding extracellular protein synthesized by many cells. The N-terminal of the protein is responsible for the anti-angiogenic effect (12). The use of full-length TSP1 has been prohibitive due to its size and other multiple biological function (9).

Caffeic acid phenethyl ester (CAPE) is a pharmacological compound extracted from propolis, a constituent of honey, which possess anti-inflammatory, immune-modulating, anti-carcinogenic and anti-oxidant properties (7,8). Treatments with CAPE showed that CAPE inhibited angiogenesis by inhibiting the productions of MMP and VEGF, preventing VEGF expression and reducing neovascularization (10,11,12,14).

In order to understand the mechanism in detail, we examined the relationship between VEGF, MMP, Endostatin and Thrombospondin-1 levels and the therapeutic dose of CAPE in a gastric cancer cell line.

MATERIAL and METHOD

Cell culture

Gastric cancer cell line NCI-N87 was incubated with DMEM F-12, 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin at 37°C in an incubator supplied with 5% CO₂. Cells were seeded onto 96-well plates with 45,000 cell / mL in each well. Once the cells adhered to the surface and multiplied, 1, 0.25, 0.06, 0.015 and 0.007 µg/mL from the stock solution of CAPE were added. Cell proliferation and cytotoxicity were examined 48 hours later by the MTT method (14).

CAPE administration

1 mM stock solution of CAPE was prepared by dissolving CAPE in 0.5 mM DMSO and adding 9.5 mM of medium. Using this stock solution, CAPE was administered at 1, 0.50, 0.25, 0.12, and 0.06 µg/ml concentrations to the cancer cell line (15).

MTT

Mitochondrial functions of the cells and viable cell density were determined by MTT test. This test is based on a redox reaction that converts yellow MTT reagent to blue/violet formazan in mitochondria. Cells were incubated with 0.5 mg/mL of MTT in the last four hours of the culture, followed by removal of the medium. Formazan salts were dissolved in dimethylsulfoxide (DMSO) and absorbance at 570 nm was read by a multiplate UV-visible spectrophotometer (15).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde solution in PBS (pH: 7.4) and washed with PBS three times for 5 minutes each. They were incubated in 0.5% trypsin solution for five minutes and washed once again with PBS as detailed above. Cells were incubated in 3% hydrogen peroxide (H₂O₂) for 30 minutes, blocking solution for one hour, and anti-VEGF primary antibodies for 18 hours. Following washing, sections were stained with biotinylated anti-mouse/antihuman conjugated streptavidin-horseradish peroxidase for 30 minutes (85-9043, Zymed Histostain kit San Francisco, USA). Each secondary antibody was washed three times with PBS for five minutes. To make immunoreactivity visible, sections were developed in diaminobenzidine (DAB, 00-2020, Zymed, San Francisco, USA) for five minutes. Primary antibody was replaced by PBS for negative control. Following washing with distilled water, the sheets were mounted with mounting (00-8030, Histomount mounting solution, San Francisco, ABD) solution (13).

TUNEL

A DeadEnd Colorimetric TUNEL system kit (G7130, Promega, USA) was used for this method. Cells were fixed in 4% paraformaldehyde for 10 minutes, rinsed in three changes of buffer solution for five minutes each, treated with 4% paraformaldehyde for five minutes for a second time, rinsed in buffer solution for five minutes and incubated with TdT-enzyme solution at 37°C for one hour. Cells were treated with SSC solution containing 22% NaCl and 11% sodium citrate for 10 minutes, rinsed in buffer solution and treated with 3% H₂O₂ (TA-015-HP, Lab vision, Fremont, CA) for five minutes to inhibit endogenous peroxidase. Cells were rinsed in buffer solution at room temperature for 10 minutes, incubated with anti-streptavidin-peroxidase enzyme for 30 minutes, rinsed with buffer solution, stained with diaminobenzidine (DAB), rinsed in distilled water three times for five minutes each, mounted with mounting solution (Histomount mounting solution) and examined under a microscope (15).

RNA isolation protocol

In this study, in the control group and CAPE administered group two types of cells were used. Tripur isolation reagent was added to the cells in the flask and the scraped cells were added to the tubes. Then they were centrifuged at

3.000 rpm for 30 sec. The cells were taken to the special eppendorf tubes with beads at the bottom. They were rotated at MAGNA Lyser homogenizer at 3000 rpm for 30 seconds. The removed tubes from the homogenizer enizer were taken to the cooling block and allowed to stay at room temperature for 5 min. Then 200 microliters of chloroform were added to the tubes, incubated for 5 min at 4°C for 20 minutes and centrifuged at 12.000 rpm. This process results in DNA, RNA and protein was obtained as 3 phases. 1st phase (aqueous phase): includes RNA, colorless. 2nd Phase: contains DNA, is white. Phases 3 (organic phase); includes proteins, is red. For RNA isolation, it was put into 500 microliter colorless phase 1 tubes. 500 microliters of isopropanol were added on. They were incubated for 10 min at room temperature. They were centrifuged 10 min at 12.000 rpm at 4°C. 1 ml 75 % of ethanol was added on the precipitated form. They were centrifuged at 4°C for 5 min at 12.000 rpm. At the end of centrifugation, the supernatant was discarded. 57°C ethanol evaporated. Pipetage was done by adding 50–100 microliters of RNA free water to the remaining precipitate. Precipitation was dissolved (16).

cDNA synthesis

After addition of RNase-free water, the absorbance of the cells was measured. For each sample, a mixture of total 11.4 microlitre was prepared by 9.4 microliters of RNA + H₂O and 2 microliters by random hexamer primer. This mixture was taken into smaller tubes and pipetage was performed. Afterwards the tubes were placed in Thermal cycler. It was incubated at 65°C for 10 min. Mastermix was prepared in the meantime. For each sample, 4 microliters of the reaction buffer, dNTP 2 microliters, 1 microliter DTT, 1.1 microliters enzyme, 0.5 microliters of RNase inhibitor was prepared for a total of 8.6 microliters mastermix. Prepared mastermix (8.6 microliters) was added to the samples (11.4 microliters) taken from thermal cycler and the pipetage was performed. Final volume of cDNA samples was completed to 20 µl. Then, the tubes were placed in a thermal cycler and run according to the schedule below; at 55°C and 85 °C for 30 min 5 min (16).

Real time PCR process

cDNA sample was prepared so that the final volume of the reaction mixture was 10 µl. For each sample, 3.5 ml of dH₂O, 0.5 ml of the probe primer mix, 5 ml of 9 ml of enzyme mixture were to be obtained. To this mixture, 1 µl

cDNA sample was added and pipetage was performed. The reaction mixture was distributed to in each well of 96-well plate in amount of 10 µl PCR and was also continued to read. After one hour reading activity took place in PCR (16).

Statistical analysis

SPSS for Windows v15.0 was used to analyze the data obtained during the study. The significance of the differences between groups was tested by Mann Whitney-U test. The level of significance was set at p<0.05.

RESULTS

The gastric cancer cell line was thawed according to the protocol and transferred into flasks. We observed that the passaged cells were growing normally and prepared them for the MTT test. The cells exhibited strong adhesion. Even when we tried to separate the cells, 3-5 cells adhered to each other and formed aggregates, and this was uniform throughout all areas. Cellular adhesion was retained during this semi-confluent and confluent growth. The growth of the cells was significantly slower compared to cells of other cell lines (Figure 1).

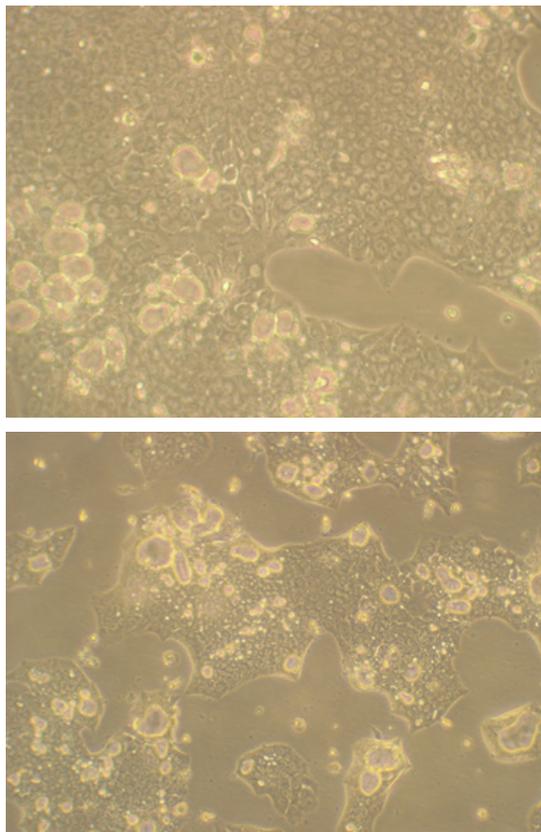


Figure 1: Photomicrograph of the gastric cancer cell line NCI-N87 before and after CAPE application showing toxic effect.

MTT confirmed that CAPE administration exerted toxic effects at various concentrations. IC50 was 0.25 µg/mL for the confluent cells while it was 0.5 µg/mL for the semiconfluent cells. There was clear cell death by the effect of CAPE which killed most of the cells at higher concentration (Figure 2).

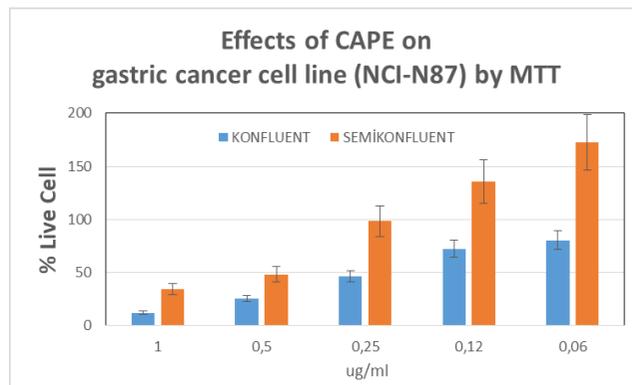


Figure 2: Toxic effects of CAPE on gastric cancer cell line NCI-N87 by MTT.

The effect of gene expressions of gastric cancer cells treated with CAPE showed that there was alteration for gene related to angiogenesis. RT-PCR analysis revealed that after the application of CAPE, endostatin protein expression values significantly (p < 0.05) increased while the value of TSP-1 and MMP protein expression significantly (p < 0.05) decreased. Moreover, decrease of VEGF protein expression was significant (Table 1).

Table 1. Statistical analysis of the expressions of angiogenic factors in the control and CAPE-administered cells. *: compared to the Control group, p<0.001

	VEGF	Endostatin	TSP-1	MMP
Control	577.2 ± 40,18	0.00000185 ± 0.00000011	0.0231 ± 0.0045	0.00000221 ± 0.00000045
Gastric cancer cell+ CAPE	490 ± 35,22	0.000025 ^a ± 0.00000006	0.00320 ^a ± 0.00511	0 ^a

DISCUSSION / CONCLUSION

Gastric cancer is amongst the most common causes of cancer-related mortality. It is the second most common form of cancer in males and the third in females. Significant

advances in diagnosis and treatment of this disease have been achieved in the last decade, but prognosis is still poor and some 50% of the patients die within two years (1,16). Gastric cancer is closely related to age. Living in the same environment is likely to play a role as well, and it is generally agreed that environmental factors facilitate genetic predisposition. In 1953, Aird reported an association between blood type A and gastric cancer: the relative risk in blood type A individuals is 1-2 times higher than in individuals with blood type O. A variety of foods play roles in the etiology of gastric cancer. Low quality diets, especially ones poor in dairy products, animal proteins and vitamins and rich in starch, have been implicated in the development of the disease (17). Despite advanced surgical techniques and standardization of multimodal therapy, survival after surgery is still low (18). Genes of cells which possess the potential to cause cancer by mutation are referred to as oncogenes. These genes control cell proliferation and invasion through angiogenesis (2). Cancer metastasizes by direct spread, by penetrating the lymphatic system or via blood vessels. Research has shown that invasion is facilitated by angiogenesis (3). In general, tumors are considered to be equipped with angiogenic capabilities and growth, invasion and metastasis depend on angiogenesis (19). Folkman in 1971 proposed a hypothesis which he had been developing since the 1960s that tumor growth was related to angiogenesis, and this initiated studies on angiogenesis pathways and molecular targets (20,21).

One of the most promising natural flavonoids is the CAPE group. This is extracted from propolis, which is produced by honey bees to protect the hive from external infectious agents. CAPE is a pharmacological compound with anti-inflammatory, immune-modulating, anti-carcinogenic and antioxidant properties (11,14). Grunberger et al. reported that CAPE had significant cytotoxic effects on various tumor cell lines (22). Lee et al. showed that CAPE prevented the invasion capability of SK-Hep1 cells and reduced the amount of MMP-9 (23). In the present study, we examined the relationship between the therapeutic dose of CAPE and VEGF, MMP, Endostatin and Thrombospondin-1 levels in a CAPE-treated cancer cell line.

We used different concentrations of CAPE on the gastric cancer cell line and quantified CAPE cytotoxicity by MTT and found that at a 0.5 µg/mL dose, CAPE exerted a cytotoxic effect on gastric cancer cells, causing them to lose viability. Tumor angiogenesis is different from normal physiological angiogenesis. The balance between

angiogenic and anti-angiogenic factors is tipped in favor of angiogenesis when the tumor tissue grows rapidly (24).

A high molecular weight (450-kDa) multi-function glycoprotein, Thrombospondin-1 (TSP), has proven anti-angiogenic properties. Despite a few publications reporting otherwise, its anti-angiogenic effects have been shown in colorectal, lung, bladder and breast cancers (27). Nakao et al. reported that TSP-1 had anti-angiogenic effects in gastric cancer (25). Ling-fang et al., on the other hand, reported that TSP inhibited angiogenesis by suppressing angiogenesis and tumor growth (26). A review of the literature did not yield any studies on the relationship between CAPE and TSP-1 while Aksoy et al. reported that CAPE lowered the increased ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin's motif) expression (27). In the present study, TSP-1 was reduced after adding to CAPE.

72 kDa MMP-2 and 92 kDa MMP-9 are secreted in a paracrine/autocrine fashion and have been shown to stimulate the release of VEGF and play key roles in tumor growth and angiogenesis (28). Zinc-dependent MMP is modulated by various growth factors, cytokines and hormones. Kanga et al. showed that MMP-9 was a key enzyme in gastric cancer metastasis (29). MMP-2, played a critical role in tumor invasion and metastasis in gastric cancer (30). Huacman et al. stated that MMP-2 and MMP-9 were related not only to the growth of gastric cancer but also degradation of the extracellular matrix and inhibition of apoptosis (28). They also reported close relations between MMP-2, MMP-9 and VEGF (28). Chih et al. reported that CAPE prevented the migration / invasion ability of SCC-9 cancer cells by inhibiting the expression and enzymatic activity of MMP-2 protein (31). Keshavarz et al. reported that CAPE inhibited MMP-9(32). Liao et al. reported that CAPE could inhibit only 47.8% of cell invasion, also it reduced VEGF and MMP-9(17). In our study, we observed that CAPE significantly reduced MMP. This result supports previous studies.

VEGF plays a pivotal role in the promotion of vascularization, not only in physical but also in malignant conditions (33). Angiogenesis induced by VEGF is an important pathological regulator in newborns (34). Yoriko et al. showed that VEGF was associated with cancer (34). VEGF was most potent angiogenic factor for metastasis and the growth of solid tumors (35). VEGF has been found at significantly high levels in gastric, lung, breast, thyroid, renal, bladder, ovarian, uterine and pancreatic cancers (34). Zhu et al. stated that abnormal interactions in the

STAT 3 pathway resulted in increased VEGF angiogenesis (35). Numerous studies have found that a potential cancer treatment can be followed via STAT3. STAT3 is very effective in inhibiting VEGF and prevents tumor angiogenesis (36). Kikuchi and colobrate observed VEGF expression when they immunostained certain malignant tissues (33). VEGF can facilitate the dissemination, growth and spread of gastric cancer cells but it is found in small quantities in the intestines and its effect is limited (33). In the Zhu et al. reported that VEGF-C was rich in gastric cancer tissue and VEGF-C was related to pathological staging (in terms of metastasis and infiltration) of the gastric tissues (37). Yonging et al showed that VEGF was positive in 81% of gastric cancer tissues (38). Basini et al. showed that CAPE exerted anti-angiogenic activity and inhibited VEGF (7). Izuta et al. argued that CAPE suppressed VEGF by showing anti-VEGF behavior (39). Omene et al. also showed that VEGF decreased with CAPE (40). Mancielli et al. observed a similar result in breast cancer cells whereby CAPE reduced VEGF expression (41). In the present study, we also found a decreased VEGF expression with CAPE.

Endostatin is a 20 kDA C-terminal fragment of collagen XVIII and an angiogenesis inhibitor produced by tumor proteases in the extracellular matrix (42). Collagen XVIII is present in extracellular matrices and its layers (42). Pufea et al. showed that endostatin inhibited neovascularization induced by VEGF (43). The angiogenic effects of the endostatin mechanism are complex. Patra et al. highlighted the importance of endostatin on the suppression of VEGF and the expression of anti-angiogenic pigments (44). Tumor growth and metastasis preventive effects of endostatin have been shown in a number of *in vivo* and *in vitro* studies. On the other hand, Szarvas et al. found that systemic endostatin concentration was elevated in several types of human cancers (45). Some researchers assessed VEGF and endostatin levels in gastric cancer patients but did not find a significant relation between endostatin and gastric cancer although VEGF expression was increased (43). Xiea et al. stated that endostatin and Thrombospondin-1 were key inhibitors of angiogenesis (46). Dönmez et al. tried to inhibit angiogenesis through natural ways by suppressing VEGF and MMP using anti-angiogenic TSP molecules (24). El-Refaei et al. stated that the endostatin level was increased by CAPE (47). Abduljawad et al. showed that CAPE increased the endostatin level (48). We found an increase in endostatin with CAPE supplementation.

Dönmez et al. reported that VEGF and TSP-1 were not significantly related. These results suggest that VEGF and TSP-1 were effective through independent mechanisms (24). Increase in TSP-1, an anti-angiogenic agent, suggests to us that VEGF and TSP-1 are effective through independent mechanisms. Some research has shown that CAPE prevents angiogenesis by inhibiting VEGF through the STAT 3 signal pathway (39,49).

The decrease in the amount of VEGF as a result of our study confirms that increase of endostatin, production of direct cell growth and migration and moreover VEGF inhibits the effect of angiogenesis (12,50). Similar to the decrease in VEGF, MMP level is also decreased. VEGF and MMP molecules are related because endothelial cells induced by VEGF initially synthesize MMPs. MMPs become free and disrupt the structure outside the blood vessels. This disruption expedites angiogenesis (3). In previous study, we examined the protein alterations of these matrix molecules immunohistochemically and measured the levels of VEGF, MMP-9, ES and TSP-1 using the ELISA test. We demonstrated the beneficial effect of CAPE on a gastric cancer cell line including inhibition of proliferation and induction of some proteins that might be related to decreased angiogenesis (51).

In conclusion, we found that CAPE, a naturally occurring substance, reduces VEGF and MMP, two important angiogenesis activators, while increasing endostatin, a potent angiogenesis inhibitor. Despite its being an angiogenesis inhibitor, an increase in the levels of TSP-1 suggested that it exerted its effects via a different mechanism from VEGF, MMP and endostatin. The changes in MMP, VEGF, TSP and endostatin signified that CAPE, a naturally occurring substance, had significant therapeutic effects on gastric cancer. In the light of these findings, we conclude that CAPE is a natural and inexpensive substance and it can be considered for the treatment of gastric cancers. However, more detailed studies are needed.

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