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ORIGINAL ARTICLE / ÖZGÜN ARAŞTIRMA

Evaluation of Bax protein in breast cancer cells treated with tannic acid

Tannik asit uygulanan meme kanseri hücrelerinde Bax proteinin değerlendirilmesi

Ahu Soyocak, Didem Turgut Coşan, Ayşe Başaran, Hasan Veysi Güneş, İrfan Değirmenci

Department of Medical Biology, Medical Faculty, Eskisehir Osmangazi University, Eskişehir- Türkiye

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ÖZET

Amaç: Bir bitki polifenolü olan Tannik Asit (TA)'in antikarsinojenik, antioksidan, antimutajenik, antimikrobiyal, antiallerjik ve antiinflamatuar aktiviteleri vardır. Bununla birlikte şimdiye kadar, meme kanserinde TA'in antikanser aktivitesinden sorumlu kesin bir mekanizma henüz açıkça tanımlanamamıştır. Bu çalışmanın amacı, insan meme kanser hücre dizisinde (MCF-7) TA'in pro-apoptotik Bax proteini üzerine etkisini araştırmaktır.

Gereç ve yöntem: Bu çalışmada meme kanser hücrelerine (MCF-7) çeşitli konsantrasyon (0, 25, 50 and 100 µM) ve saatlerde (24, 48 ve 72. saatlerde) TA uygulandı. Bu hücrelerdeki pro-apoptotik Bax protein yüzdesini belirlemek için immunohistokimyasal boyama yöntemi kullanıldı.

Bulgular: Araştırmamızın sonunda, çeşitli dozlarda TA muamelesinden sonra kanser hücrelerindeki pro-apoptotik Bax proteini yüzdesinin zamana bağımlı olarak arttığı gözlemledik. Bu artışın özellikle 72. saat'te ve 25 µM'lük dozda en yüksek düzeyde olduğu görüldü.

Sonuç: Bulgularımıza göre, TA'in Bax proteinini artırarak, meme kanseri hücrelerinin apoptoza gitmesini sağlayabileceğini düşünmekteyiz. Bu çalışmadaki sonucun desteklenebilmesi için TA'in apoptotik yoldaki diğer proteinlerle ilişkisinin ayrıntılı bir şekilde değerlendirilmesi gerekmektedir.

Anahtar kelimeler: Meme kanseri, antikarsinojenik, hücre dizisi, apoptoz, tannik asit, MCF-7, Bax proteini

ABSTRACT

Objectives: Tannic acid (TA), a plant polyphenol, is known to have anti-carcinogenic, anti-oxidant, anti-mutagenic, anti-microbial, anti-allergic, anti-inflammatory activities. However, a precise mechanism responsible for the anti-cancer activity of TA in breast cancer has not yet been clearly described. The aim of this study was to investigate the effect of TA on the pro-apoptotic Bax protein in a human breast cancer cell line (MCF-7).

Materials and methods: In this study, TA in various concentrations (0, 25, 50 and 100 µM) were administrated at various time points (24th h, 48th h and 72nd h) to breast cancer cells (MCF-7). The percentages of pro-apoptotic Bax protein in these cells were determined by immunohistochemical staining.

Results: At the completion of the study, percentages of the pro-apoptotic Bax protein in pro-apoptotic cancer cells were found to be increased in a time-dependent manner after exposure to various concentrations of TA. This increase was at highest level with the concentration of 25 µM at 72nd hour.

Conclusion: Based on our results, we suggested that TA could induce apoptosis of breast cancer cells by increasing Bax protein. Further studies evaluating the relationship of TA with other proteins that have a role in apoptotic pathways are warranted to support the findings of this study.

Key words: Breast cancer, anticarcinogenic, cell line, apoptosis, Tannic Acid, MCF-7, Bax protein

INTRODUCTION

Breast cancer is a common malignant tumor in women. Programmed cell death, which is also known as apoptosis, is related to the response and resistance to treatment in breast cancer.[1] Apoptosis plays a crucial role in the maintenance of cell homeostasis. At least two main pathways lead to apoptosis: (i) extrinsic, consisting of cell surface TNF-related family of receptors, their inhibitory counterparts (decoy receptors) and cytoplasmic adapter or death inhibitory molecules (e.g., FADD or FLIP) and (ii) intrinsic, for which mitochondrion is the hub gov-

Yazışma Adresi /Correspondence: *Dr. Didem Turgut Coşan, Eskişehir Osmangazi Universitesi, Tıp Fakültesi, Tıbbi Biyoloji A.D., 26480 Eskisehir- Türkiye* Email: dcosan@ogu.edu.tr

erned by pro- and anti-apoptotic members of the Bcl-2 family.[2-4] The Bcl-2 protein family plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli. Some proteins of this family, including Bcl-2 and Bcl-xL, inhibit programmed cell death, and others, such as Bax and Bak, promote apoptosis.[2-5] In a study, it was shown that reduced Bax levels were associated with poor response to chemotherapy and shorter overall survival in colorectal carcinoma.[6] Conversely, enhanced Bax levels were correlated with good response to chemotherapy in several cell types, in vivo.[7]

Natural products are considered to be an important source of cancer chemopreventive agents. Polyphenols can directly modulate different aspects of the apoptotic process and/or the expression of regulatory proteins, such as the down-regulation of Bcl-2 and Bcl-XL expression, and the enhanced expression of Bax and Bak.[8-10]

Tannins are natural constituents of tea, green tea, coffee, red wine, grapes, nuts and other plant products.[11,12] Plant-derived polyphenolic tannins (500-3000 Da) can be classified into two groups, as hydrolysable and condensed tannins. The hydrolysable tannins, commonly called tannic acid (TA), contain either gallotannins or ellagictannins.[13] TA polyphenol has been described as having anticarcinogenic, anti-oxidant, anti-mutagenic, antimicrobial and astringent properties.[9,11,12] TA and its structural monomer, gallic acid, are also capable of inducing apoptosis in animal cells.[14] Gallic acid acts as a pro-oxidant in the induction of apoptotic cell death in human glioblastoma cells.[15] Inhibition of the proteasome by TA in Jurkat T-cells results in accumulation of two natural proteasome substrates, namely the cyclin-dependent kinase inhibitor p27 Kip1 and the pro-apoptotic protein Bax, followed by growth arrest in G1 and induction of apoptotic cell death.[13]

In this study, we aimed to investigate effects of TA on Bax proteins in MCF-7 cell lines.

MATERIALS AND METHODS

Cell Culture and Cell Treatment

MCF-7 cells were grown in the Laboratory of Medical Biology, Eskisehir Osmangazi University, Eskisehir, Turkey. MCF-7 breast cancer cells were cultured in the flasks (25 cm^2) using RPMI 1640 (Roswell Park Memorial Institute 1640, Biological Industries Ltd., Haemek, Israel) supplement with 10% fetal calf serum (Sigma-Aldrich Inc., St. Louis, USA) and penicillin-streptomycin (Sigma-Aldrich Inc., St. Louis, USA), and were grown as monolayers in a humidified atmosphere of 5% CO_2 at 37°C.

TA (Acros Organics, New Jersey, USA) was used to treat MCF-7 cells. MCF-7 cells were plated on chamber slides (9 cm^2) and in a medium containing different concentrations of TA [0, 25, 50 and 100 μM dissolved in dimethly sulfoxide (DMSO)] for 24, 48 and 72 h.

Immunohistochemical staining assay

Bax protein in MCF-7 cell line was analyzed by immunohistochemical staining assay. Immunohistochemical staining was performed by streptavidine-biotin-peroxidase staining method with the use of immunohistochemical detection kit (Lab vision Corporation, Fremont, CA, USA).

Cells were plated on 4-well chamber slides for immunohistochemical staining. Fresh medium containing 0, 25, 50 and 100 μM TA concentrations were added to cells. Cells were incubated for 24, 48 and 72 hours and then were fixed in flasks. These fixed cells were firstly incubated with serum blocking solution (10 min) and then were incubated with Bax protein antibody (1 hour). After washing with PBS solution, they were incubated with biotin-labeled secondary antibody (10 min) and streptavidin-peroxidase conjugate (10 min). Thereafter, they were treated with substrat-chromogen solution (AEC) until staining was seen, after which cells were exposed to hematoxylin for 1 to 2 minutes. Preparations were mounted with mounting solution and evaluated under the microscope in a double-blind manner. For immunohistochemical index calculation, values that were obtained by counting the stained cells in five different randomly selected areas in each of the three different preparations (approximately 100 cells in each of the areas) were formulated as follows:[16]

Immunohistochemical index: (Staining cells/ Total cells) x 100

Statistical Analysis

Data were expressed as means ± S.E. The comparisons between the groups were performed using one way variance (One way ANOVA) analysis, the

Dunnett's were used as the multiple comparison test and $p<0.05$ was considered significant. All analyses were performed using SPSS 15.0.

RESULTS

Our results showed that apoptotic Bax protein percentage increased when TA was used at a concentration of 25 µM in the 24 hour group ($p<0.001$) ($p=0.001$); at 100 µM concentration in the 48 hour group ($p<0.01$)($p=0.009$); and at 25 and 50 ($p<0.001$) ($p=0.000$), and 100 µM ($p<0.01$) ($p=0.007$) concentrations in the 72 hour group compared with the control group (Table 1, Graph 1A). Figure 1 shows the percentage of Bax protein after exposure to TA. Compared to control group (Figure 1A), most significant increase was in all concentrations at 72nd hour and among them at the concentration of 25 µM (Figure 1B). When results were interpreted with regard to relationship between TA and Bax protein, although there was a time-dependent increase, a dose-dependent relationship was not evident (Graph 1B).

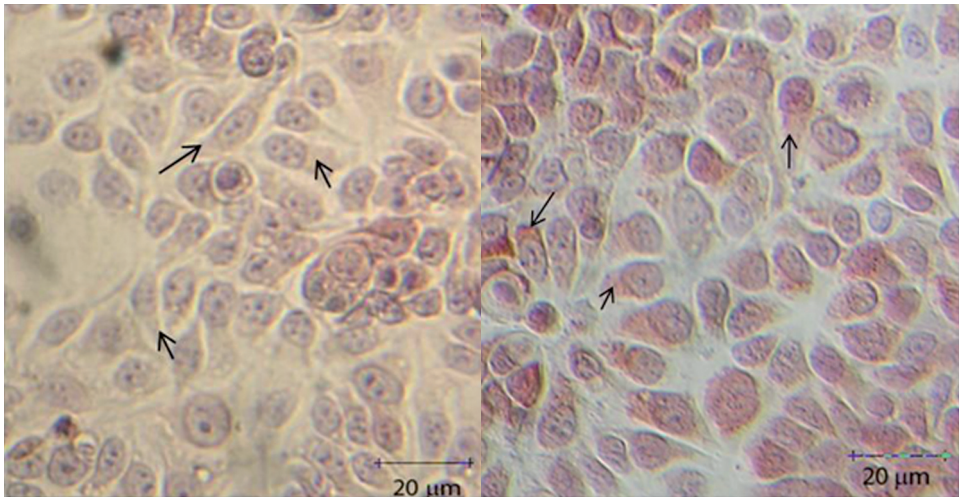


Figure 1. The immunohistochemical images of Bax protein on MCF-7 cell line in control (A) and 72nd hour 25 µM tannic acid ($p<0.001$) groups. Red-colored staining around nucleus (arrows) represents Bax protein in the preparation (B).

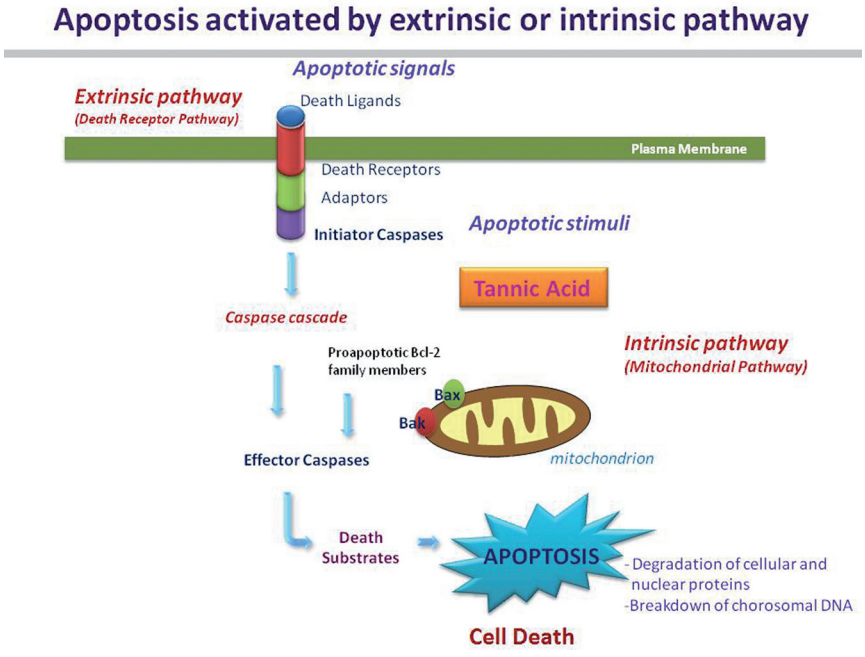
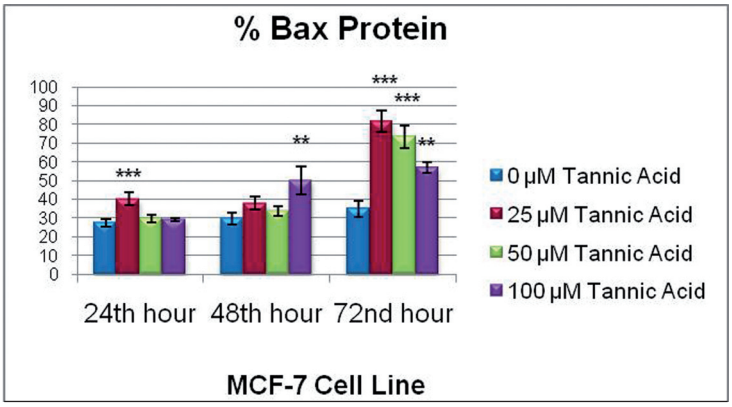
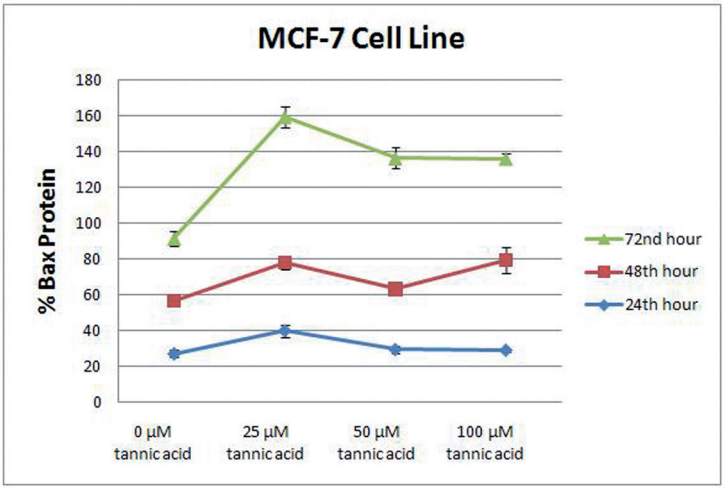


Figure 2. The effect of TA in intrinsic pathway on Bax protein



A *p<0.05,** p<0.01,*** p<0.001, n.s.(not significant)



B *p<0.05,** p<0.01,*** p<0.001, n.s.(not significant)

Graphics 1. (A) Percentage of Bax protein calculated from the control group. (B) dose- and time-dependent effects of TA on Bax protein.

Table 1. The Bax percentages of the MCF-7 cell line groups by hours and concentrations.

Groups	Concentrations (µM)	MCF-7 % Bax Ratio		
		24 h	48 h	72 h
Control Group (C)	0 (DMSO)	27.27±2.00	29.67±3.23	34.83±4.13
	25	40.15±3.34	37.87±3.36	81.70±5.66
Tannic Acid (TA)	50	29.62±2.07	33.77±2.75	73.28±5.89
	100	29.27±0.88	50.25±7.45	56.92±2.76
Statistical Analysis		$C\text{-}TA_{25}$ *** p=0.001	$C\text{-}TA_{25}$ n.s p=0.458	$C\text{-}TA_{25}$*** P<0.001
		$C\text{-}TA_{50}$ n.s p=0.803	$C\text{-}TA_{50}$ n.s p=0.865	$C\text{-}TA_{50}$*** P<0.001
		$C\text{-}TA_{100}$ n.s p=0.864	$C\text{-}TA_{100}$ ** p=0.009	$C\text{-}TA_{100}$ ** P=0.007

*p<0.05,** p<0.01,*** p<0.001, n.s.(not significant) n=15 (5 each area selected from 3 different preparations)

DISCUSSION

Bcl-2 family proteins are important regulators of the apoptosis signaling pathway (Figure 2). There are studies investigating the effects of many natural compounds on MCF-7 cell line, but a few studies with tannins. Increasing evidence supports the hypothesis that TA, a plant polyphenol, exerts anti-carcinogenic activity in chemically induced cancers. In the present study, we also showed that TA treatment may induce an increase in Bax expression.

In a study, it has been shown that TA induced apoptotic death in acute myeloid leukemia (AML) HL-60 cells in a dose- and time-dependent manner, as well as increase of sub G1 fraction, chromosome condensation, and DNA fragmentation. Also, TA induced the expression of the pro-apoptotic protein, Bax, in HL-60 cells after 24 hours of treatment with TA.[17] In another study on HepG2 liver cell line, epigallocatechin-3-gallate (EGCG), a tea polyphenol that is a similar to TA, increased Bak and Bax proteins at different doses and different hours in a dose-dependent manner.[9] In another study, it has been shown that decreased levels of Bax protein correlated with increased levels of Bax degradation in aggressive human prostate cancer.[18]

In the studies concerning TA and apoptosis 2, 6, 12 and 24 μM condensed tannin were administrated to normal fibroblast lung (HEL 299), colon (CaCo-2), breast (MCF-7, Hs578T) and prostate (DU 145) cells. After 24 hours, normal cells were alive but the cancer cell death was increased.[19] In another study performed with prostate cancer cell line (LNCaP), 5 and 10μmol/L TA significantly increased apoptotic index at 72nd hour as compared to control group.[20] In another study performed on human Jurkat T cells, 50 and 100 μg/mL TA increased apoptotic cell death in a dose-dependent manner at 24th hour.[13] As is seen in all of these studies, the effects of TA are different at various concentrations and hours in cell lines. When these differences were considered, there were no dose-dependent Bax protein increase in the present study. However, there was an increase in percentage of Bax protein with elapsing of time. Most significant increase was seen at 72nd hour in a concentration of 25 μM.

Bax protein in breast tumor cells might be contributed to the previously established anti-carcinogenic activity of TA. These studies also evidenced that increase of Bax protein is one of the possible mechanisms. Therefore, the pro-apoptotic Bax protein may be the target of TA in cancer prevention.

Before establishing TA as a cancer chemopreventive and/or anticarcinogenic agent, further detailed studies are needed. Besides the present study evaluating the effects of TA on Bax protein in the breast cancer cells is also necessary to evaluate its relationship with other proteins that have a role in apoptotic pathway and their relationships with each other.

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