EXPERIMENTAL STUDY

Apoptosis of colon cancer cells under the effect of geldanamycin derivate

Kosova F[1], Kasar Z[2], Tuglu I[3], Ozdal Kurt F[4], Gok S[5], Ari Z[6], Imren T[1]

Faculty of Health Science, Celal Bayar University, Manisa, Turkey. **fundakosova@gmail.com**

ABSTRACT

AIM: The apoptotic effect of geldanamycin derivative may be important for the colorectal cancer therapy. The mechanisms of apoptosis require understanding of the behavior of colon cancer cell line Colo-205 which mimics colon adenocarcinoma. Therefore, the effect of IC50 dose of 17-allylamino-17-demethoxygeldanamycin (17-AAG) on the colon cancer cells in vitro was studied for its anti-apoptotic activity.

METHOD: Apoptotic ratio of the Colo-205 cells was determined after 17-AAG application with terminal deoxy-nucleotidyl transferase dUTP nick end labeling (TUNEL) staining and apoptosis related genes. Apoptosis signal path related key mitochondrial proteins, cytochrome c, bcl-2, caspase 9 and Apaf-1 expression were examined with RT-PCR method.

RESULTS: 17-AAG caused induction of cell death. Apoptotic related genes such as cytochrome-c, Apaf-1 and caspase-9 protein expressions were increased significantly ($p < 0.05$) and anti-apoptotic bcl-2 expression was decreased significantly ($p < 0.05$). Our results indicated that the application of 17-AAG on Colo-205 cells showed anticancer effect by the apoptosis due to alteration of apoptotic genes.

CONCLUSION: The apoptotic effect of 17-AAG as an natural product for alternative medicine would be very important for the success and quality of life during the treatment of colon carcinoma with the combination of anticancer drugs *(Tab. 1, Fig. 2, Ref. 32)*. Text in PDF *www.elis.sk*.

KEY WORDS: colon cancer, 17-AAG, apoptosis, RT-PCR, in vitro.

Introduction

Cancer is a disease caused by changes in the critical genes that control cell proliferation, differentiation, survival and apoptosis. Colon cancer stays in third place among the most common types of cancer (1). Many people are diagnosed with colon cancer in the world and most of them die with many complication (2). The prevention of this disease and even at early diagnosis stage is still very difficult with current treatment options. Apoptotic cell death is an important mechanism and target for the anti-cancer treatment (3, 4).

The mitochondria-mediated apoptosis occurs with the release of cytochrome-c to cytosol where it is activated. Cytochrome c, cytosolic apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9 assemble to form apoptosom. Procaspase 9 turns into this complex to caspase 9 and caspase 9 enables activation of caspase 3 and 7 (5, 6). Thus, cell death occurs (6). One of the most important mechanism of apoptosis modulators is Bcl-2 gene. Bcl-2 is the first gene that is determined to protect cells from apoptosis (7). It shows this anti-apoptotic effect by preventing cytochrome c release and the activation of effector protease. Reduction in the levels of Bcl-2 cells leads to apoptosis and its increase prevents cells from dying (8).

Signaling proteins which have an important task in the initiation and progression of apoptosis are called chaperone. Heat shock proteins as molecular chaperone function in the regulation of cellular homeostasis and cell survival (9). HSP-90, which is a heat shock protein, plays a role in regulating apoptosis by interacting with different proteins at critical control points and prevents apoptosis due to its cell protective property (10). The inhibition of HSP-90 with various agents causes the release of cytochrome c cytosol which plays an important role in the initiation of apoptosis by causing mitochondrial membrane depolarization (11). Geldanamycin, an HSP-90 inhibitor and its derivatives, has apoptosis-inducing effects. Geldanamycin is ansamisin benzoquinone antibiotic which displays anti-carcinogenic effect. 17-AAG is a derivative of geldanamycin. It has been shown that 17-AAG induces apoptosis in colon cancer cell lines by blocking the function of chaperone HSP-90 (12–15). However, the relation of this effect with alteration of related genes is not known very well.

In light of these information, this study aims to investigate the effect of 17-AAG on colon cancer cell line for anti-apoptotic effects due to alterations of related genes.

[1]Faculty of Health Science, Celal Bayar University, Manisa, Turkey, [2]Department of Chemistry, Celal Bayar University Science and Art Faculty, Manisa, Turkey, [3]Department of Histology and Embryology, Celal Bayar University Medical Faculty, Manisa, Turkey, [4]Department of Biology, Celal Bayar University Science and Art Faculty, Manisa, Turkey, [5]Department of Pharmacology, Celal Bayar University Medical Faculty, Manisa, Turkey, and [6]Department of Medical Biochemistry, Celal Bayar University Medical Faculty, Manisa, Turkey

Address for correspondence: F. Kosova, MD, Faculty of Health Science, Celal Bayar University, Manisa, Turkey.
Phone: +090.5335573629

Material and methods

Cell culture and 17-AAG application

Colon cancer cell line Colo-205 was bought from the firm ATCC and was dissolved in accordance with the protocol and then was taken into flasks. Colo-205 cell line was cultured in DMEM F-12, 10 % FCS, 1 % L-glutamine and 1 % penisilin-streptomycine containing cultural medium at 37 °C and in 5 % CO_2 incubator. 17-AAG, a geldanamycin derivative, was prepared in stock solution as 10 mM by dissolving in DMSO solution. Then this prepared stock solution was performed on different cancer cell lines for treatment with 1, 3, 10, 100 and 500 µM doses of 17-AAG to found IC50 which was 8.06 uM (16).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Dead End Colorimetric TUNEL system kit (G7130, Promega, USA) was used for this technique. After the cells had been incubated with 4 % paraformaldehyde for 10 min, the cells were washed with buffer solution for three times 5 minutes each. After being performed and washed with 4 % paraformaldehyde, the cells that were washed with buffer solution for 5 minutes are incubated for an hour with TdT-enzyme solution at 37 °C. The cells were applied 3 % H_2O_2 (TA-015-HP, Lab Vision, Fremont, CA) for five minutes after they are applied 22 % NaCl and 11 % sodium citrate washed for ten minutes and washed with buffer solution. The cells were washed with buffer solution for 10 minutes at room temperature and were incubated with anti-streptavidin-peroxidase enzyme for 30 minutes. The cells that were washed with buffer solution were dyed with diaminobenzidine (DAB). The cells were washed 3 times for 5 minutes in distilled water. Then they were examined under a microscope by being closed with histomount mounting solution (17).

RNA isolation protocol

In this study, in the control group and 17-AAG 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 homog-

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 (18).

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 + H_2O and 2 microliters by random hexamer primer. This mixture was taken into smaller tubes and pipetage was performed. Afterwards the tubes were placed in Thermalcycler. 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 thermalcycler 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 (18).

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 dH_2O, 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 (18).
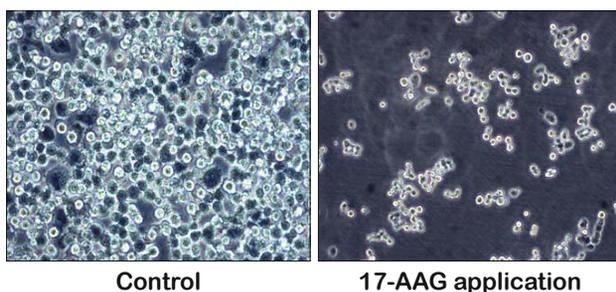

Control   **17-AAG application**

Fig. 1. Cells that are semi-confluent and confluent in Colo 205 colon cancer cell lines.

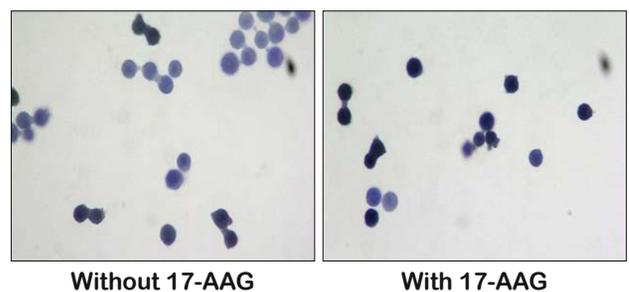**Without 17-AAG**   **With 17-AAG**

Fig. 2. Images of the cells according to the TUNEL method.

Tab. 1. The values of expressions of apoptotic factors on Colo 205 cell lines after treatment with 17-AAG by RT-PCR analysis.

	CytochromE	Bcl-2	Apaf-1	Caspase 9
Control	0.00255000±0.00085000	0.00020100±0.00015000	0.00024300±0.00065000	0.00053200±0.00025000
17AAG	0.00517000[a]±0.00155000	0.00018400[a]±0.00015000	0.00051600[a]±0.00035000	0.00107000[a]±0.00045000

[a] – when compared relative to control p < 0.05

Statistical methods

SPSS 15.0 statistical program was used to evaluate the data obtained from the experiments. The significance of differences between groups was evaluated by Mann–Whitney U test. The level of significance was accepted as p < 0.05 (19).

Results

The passaged cells of Colo-205 were observed as small and round shape with weak and late adhesion to the tissue culture plastic. They showed spiny protrusions with stick to other cells and they preserved their roundness. They were rarely viewed as bipolar morphology. There was clear death effect of 17-AAG which killed most of the cells (Fig. 1).

There was apoptotic cell death after the application of 17-AAG at IC50 dose which was 8.06 uM (Fig. 2). The cells with smaller, chromatin condensation, bleb and protrusions morphology were observed by the toxic effect of 17-AAG. Apoptotic index was changed from 1.2 ± 0.6 to 2.5 ± 0.5 under the effect of 17-AAG.

The effect of gene expressions colon cancer cells treated with 17-AAG. Treatment doses are looked with RT-PCR analysis (Tab. 1). After the application of cytochrome c, apaf-1, caspase 9 protein expression values significantly (p < 0.05) increased while the value of bcl-2 expression significant (p < 0.05) decreased.

Discussion

Colon cancer is one of the most common malignancies which was the third reason for the annual worldwide cancer related deaths with 1 million new cases and more than 600.000 annual death estimates (5). Apoptosis is a programmed cell death which is regulated by many proteins (20). Apoptosis is a target for the cancer treatment (4). It has been shown that apoptosis is associated with the elimination of the potential cancer cells and tumor growth (21). In this study, we showed the induction of apoptosis in the colon cancer cell line with the alterations of apoptosis related genes. Similarly, previous study has shown increase of mitochondria related and death receptor-mediated apoptosis in Colo-205 cells (22).

HSP-90, a heat shock protein, inhibits apoptotic response due to its cellular protective nature. Geldanamycin and its derivatives play an important role in inhibiting of HSP-90. 17-AAG is the first HSP-90 inhibitor which prevents its protective feature and provides the occurrence of apoptosis in cells. This study and previous studies showed that 17AAG has significant apoptotic and cytostatic activity on colon cancer (12, 15). 17-AAG binding to HSP-90 with the provision of destruction oncogenic proteins such as Raf-1 and Akt caused cell cycle arrest and apoptosis in different type of colon cancer cell lines (HCT116, HCT15, HT29) by blocking the signal transmission (13, 14).

Bcl-2 plays an significant role in the occurrence of apoptosis. The reduction of the bcl-2 has been reported to inhibit the mitochondrial release of cytochrome c of bcl-2, caspase activation and the prevention of anti-apoptotic effect (23). In our study, bcl-2 expressions was decreased significantly after 17-AAG treatment. This observation was supported by the previous studies. Similar results have been also shown by the studies of acute myleiod leukemia (24) and breast cancer cell line (25). The application of bcl-2 antagonist with 17-AAG caused anti-tumor activity in the cancer cells resistant to apoptosis due to high levels of bcl-2 (26).

Apoptotic cell death in the cells that were inhibited by HSP–90 takes place by the release of cytochrome c to cytosol by mitochondrial way (27). In our study, it was shown that the expression of cytochrome-c was increased statistically significantly after treated with the 17-AAG on the colon cancer cell line. It was similar to previous studies where there was a decrease of bcl-2 expression and an increase of the cytochrome c and caspase 9 expressions (26, 28).

It has been reported that apaf-1 and caspase 9 are required for the cytochrome-c induced apoptosis (29). We found in our study that apaf-1 expression was increased significantly after 17-AAG application in colon cancer cell line and in HL-60 cell line (30).

Caspases are the central components of apoptotic program with an active role (31). In this study, it was determined that caspase 9 increased statistically significantly on colon cancer cell line when treated with 17-AAG. This was similar to apoptotic cell death in bladder cancer cells (31) and lymphoma cells (32) in which 17-AAG-induced cell death increased expression of important elements of apoptosis such as caspase 9 and caspase 3.

In conclusion, the application of 17-AAG on colon cancer line caused induction of apotosis with the statistically significant expressions of apoptotic proteins such as cytochrome c, apaf-1, caspase 9 and reduction of anti-apoptotic protein bcl-2. Therefore, the application of 17-AAG appears to to contribute to the treatment of colon cancer. By the help of natural product 17-AAG to the colon cancer treatment may help survival of cancer patients.

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