Cellular and Molecular Biology

CMB Association Publisher

Journal homepage: www.cellmolbiol.org

Effects of 7,12-Dimethylbenz(a)anthracene on Apoptosis of Breast Cancer Cells through Regulating Expressions of FasL and Bcl-2

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ARTICLE INFO

Original paper

Article history:
Received: October 08, 2021
Accepted: January 21, 2022
Published: January 30, 2022

Keywords:
DMBA; FasL gene; Bcl-2 gene; BC cell; apoptosis

ABSTRACT

To provides a reference basis for the apoptosis of breast cancer (BC) cells and the carcinogenesis of BC, the effects of 7, 12-dimethylbenz (a) anthracene (DMBA) on apoptosis regulators FasL and B-cell lymphoma-2 (Bcl-2) were investigated. In this study, 62 female C57BL/6 mice aged from 4 to 6 weeks were randomly divided into control group (CG) and test group (TG), with 31 mice in each group. The TG was given DMBA solution by gavage at a dose of 50 mg/kg, and the CG was given normal saline of equal volume. On the second day after the experiment, all the mice were killed by cervical dislocation. The morphology of the mammary gland was observed by hematoxylin-eosin (HE) staining, and the differences of FasL and Bcl-2 protein expression (PE) were detected by immunohistochemistry. The mRNA expression levels of FasL and Bcl-2 were detected by quantitative real-time PCR (qPCR). Breast cell apoptosis status of mice in the two groups was detected by the terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL) method. The results showed that after HE staining, the tumor cells in the TG were stacked up to form a substantial structure. The expression level of FasL protein in the CG was greatly lower than that in the TG, and the positive rate (PR) was 20.25%, which was greatly lower than that of 89.65% in the TG (P<0.01). The expression level of Bcl-2 protein in the mammary gland tissues (MGTs) of mice in the TG was greatly higher than that of the CG, and its PR was 87.96%, which was greatly higher than that of 31.48% in the CG (P<0.01). The expression levels of FasL mRNA in the MGTs of mice in the TG and CG were 5.82±4.37 and 1.27±0.12, respectively, and there was a statistically obvious difference (P<0.05). The mRNA expression levels of Bcl-2 in the TG and the CG were 18.97±2.65 and 2.02±0.54, respectively, and there was an extremely obvious difference (P<0.01). The apoptosis rate of mammary gland cells in the TG was (19.79±3.53) %, and that in the CG was (2.93±0.28) %, and there was an extremely obvious difference (P<0.01). It indicated that DMBA inhibited the apoptosis of BC cells by regulating the up-regulation of FasL and Bcl-2 expression.

DOI: http://dx.doi.org/10.14715/cmb/2022.68.1.24

Introduction

BC is one of the malignant tumors that harm female health in recent years and is with the highest incidence among female malignant tumors (1). According to statistics, nearly 1 million BC patients are diagnosed each year, and 60% of them die from the disease (2). The carcinogenesis of tumors results in abnormal cell proliferation and death. Current studies show that the inhibition of apoptosis has a significant correlation with the carcinogenesis and progression of malignant tumors (3). Apoptosis is not only important in maintaining normal morphology and functions of tissues and organs, but also is related to physiological processes such as growth, development, reproductive aging, etc. And it is related to the carcinogenesis of malignant tumors, carcinogenesis of proliferative diseases, and other pathological phenomena (4).

With the continuous development of cell molecular technology, researchers have found that the genes of the Bcl-2 family are involved in the regulation of apoptosis (5), which is the central regulator of programmed cell death and one of the key factors in the regulation of apoptosis. Bcl-2 gene is a representative apoptotic inhibiting factor in the Bcl-2 family (6), which not only delays tumor proliferation but is positively correlated with physiological behaviors such as low cell proliferation rate and less cell necrosis (7). Many studies have found that the expression of the Bcl-2 gene is related to the development of gastric cancer, pancreatic cancer, BC, and other tumors (8). Bcl-2 can resist cell death in a variety of ways and is recognized as an anti-

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apoptotic factor currently (9). Fas/FasL is a pair of proteins mediating apoptosis. FasL, also known as the death factor, is a transmembrane protein in the tumor necrosis factor (TNF) family (10), whose mechanism is mainly to regulate apoptosis by activating the expression of T lymphocytes and NK cells. Studies have found that when FasL binds to the receptor Fas, a variety of proteases or endogenous endonuclease are activated, causing apoptosis of cells that express Fas, transmitting the signal to the inner cell membrane, and eventually leading to programmed cell death (11). FasL expression varies greatly among different types of tumors, whose expression is higher in lymphatic system-related cancers and greatly high in BC, liver cancer, and other non-lymphatic system tumors (12).

DMBA is a kind of polycyclic aromatic hydrocarbon, which is a lipophilic carcinogen. It can induce multiple tumors such as BC and leukemia, ovarian cancer and leukemia, etc. Many researchers often use DMBA to establish animal models of BC or leukemia through gavage, local application, or subcutaneous injection. Studies have found that a DMBA dose of 15mg can induce 100% breast tumor in rats (13). Other studies have found that there are many similarities between DMBA induced BC and human BC in tumor tissue morphology, tumor origin, and hormone-dependent of tumor (14). But the pathogenesis of BC is still unclear. Although DMBA is often used as a modeling drug for animal BC research, there are few studies on the effect of DMBA on the FasL gene and Bcl-2 gene expression and the relationship between DMBA and apoptosis of BC cells.

Based on the current lack of studies on the influence of DMBA on the FasL gene and Bcl-2 gene expression, DMBA was used as a modeling drug for BC to explore the influence of DMBA on the FasL gene and Bcl-2 gene expression and the relationship between DMBA and apoptosis of BC cells.

Materials and methods

Experimental Animals and Grouping

62 female C57BL/6 mice aged from 4 to 6 weeks, weighing 20-25g, were kept in a clean laboratory animal room with a room temperature of 25°C and relative humidity of about 55%. Under 12 hours of light, they could drink and eat freely. After 2 weeks of adaptive feeding, all mice were randomly divided into CG and TG, with 31 mice in each group. The TG was given DMBA solution at a dose of 50 mg/kg once a week for 5 weeks, and the CG was given normal saline of equal volume. The mice in both groups were raised in the normal way. All animal experiments in this study were approved by the experimental animal management committee and the experimental methods were conducted according to the approval guidelines.

Morphological Observation of Mice MGT

The morphology of mice mammary glands was observed after HE staining. On the second day after the experiment, all mice were sacrificed by cervical dislocation, and the mammary gland of the mice was completely removed. The second pair of mammary gland*s* of the mice were taken, fixed by formalin, embedded in paraffin, and sliced in 4um continuously. The obtained sections were dewaxed with xylene for 30min, hydrated with different alcohol gradients for 50min, and washed with haematoxylin for 20min. After washing, the sections were dyed with eosin for 2min, dehydrated with different gradient alcohol for 30min, and xylene was used for transparency for 20min. Neutral gum was used to seal the sections, and the pathomorphological diagnostic criteria referred to the conventional criteria (15).

PE of FasL and Bcl-2 Detected by Immunohistochemistry

10 mammary tissue specimens were taken from each group, and the PE of FasL and Bcl-2 was detected by the immunohistochemical SP method. Anti-Fasl and anti- Bcl-2 antibodies were used as primary antibodies. The SP method was operated according to the normal operation (16). The expression of FasL and Bcl-2 protein detected by the SP method was as follows. Under 400× microscopic observation, the brown-yellow staining of FasL and Bcl-2 cytoplasm or cell membrane was positive, and the average proportion of positive cells was the PR.

mRNA Expression of FasL and Bcl-2 Detected by qPCR

Total RNA was extracted from mice BC cells. Trizol was used for RNA extraction, and the specific operation methods were as follows. After the BC cells were ground with liquid nitrogen, 1mL of Trizol was added and the cells were centrifuged for 5min at 12000r/min; the supernatant was taken and mixed with 200uL of chloroform for oscillation for 15s; after being placed at room temperature for 10min, 600μL of isopropyl alcohol was added after centrifugation; after centrifugation, the supernatant was discarded, washed with 75% ethanol, dried, and dissolved in DEPC. The Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR reverse transcription kit (Takara) was used. The quantitative primer of FasL was synthesized, the sequence was Fas-F: GATGACCGTCTTGGCTGT CC; Fas-R: CATCGCTGAACGCTACTGGG; and the quantitative primer of FasL was synthesized, the sequence was Bcl-2-F: TGGCTGTCTCTGAAGAC GCT; Bcl-2-R: CTGCTGACCTCACTTGTGGC. The GAPDH gene of mice was selected as an internal reference. The primers of the GAPDH gene for amplification were F_m: AGTTCAACGGCACAGTC AAGG and R_m: CAGCCTTCTCCATGGTGGTG. Each sample was amplificated with the above three pairs of primers, and each reaction was repeated 3 times. The annealing temperature was 60°C, and the annealing time was 30 seconds, with 30 cycles. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative expression quantity (17).

Culture Method of Mice BC Cells

Mouse BC cells 4T1 were purchased from the cell bank of Shanghai institute of cell biology, the Chinese academy of sciences. The medium was rpmi-1640 culture medium, penicillin and streptomycin were added as double antibodies, and 10% fetal calf serum was added at the same time. The cells were cultured in a constant temperature incubator at 37°C and 5% CO_2 for 3 to 4 days for subculture.

Detection of Apoptosis in BC Cells

TUNEL method was used to detect the apoptosis of BC cells in mice (18). The specific operation methods were as follows. The embedded tissue sections were washed with xylene twice, 5min for each time; 95% and 75% ethanol were used for washing for 2 twice each, 5min for each time; after washing with PBS, protease K was added to remove the histones; the sections were rinsed with distilled water and added to PBS solution containing 2% H_2O_2 for a reaction for 5min; after the sections were washed with PBS, TdT enzyme buffer was added, after 3min of reaction, the TdT enzyme reaction solution was added for reaction at 37°C for 1h, and the terminational solution was added to terminate the reaction. After washing with PBS, anti-digoxin antibody labeled by peroxidase was added for 30min of reaction, then the sections were washed with PBS, 0.05%DAB color reagent was added; after washing with distilled water, methyl green was added for re-dyeing. After the sections were cleaned with distilled water, xylene was used for dehydration, sealed and dried, and fluorescence was observed under fluorescence microscope. The apoptosis rate was the proportion of apoptotic cells, and the calculation method was AI = (number of apoptotic cells/total number of tumor cells) × 100%.

Statistical Method

The data statistics were analyzed using SPSS 19.0. The mean plus or minus standard deviation ($\bar{x}\pm s$) was used to express measurement data, and the enumeration data were expressed as percentages. The measurement data conforming to normal distribution were tested by t-test, and those not conforming to normal distribution were tested by the Wilcoxon test. $P<0.05$ indicated that there was a statistically significant difference.

Results and discussion

HE Staining for Comparing Morphology of Mice MGT in Two Groups

After the two groups of mice were sacrificed by cervical dislocation, their MGTs were taken and observed under a microscope after HE staining. The results were shown in figure 1. Under the 40× microscopic observation, compared with the CG, the tumor cells in the TG were arranged into a substantial structure, separated by multiple fibers. Under 100 × microscopic observation, the tumor cells formed microcapsules and microtubules, the nuclei were smaller than those of the CG, the nucleoli were clear, and the cytoplasm secretions were stained slightly. Under 400× microscopic observation, there are abundant eosinophilic and basophilic secretions within the tumor cells and between the microcapsule spaces.

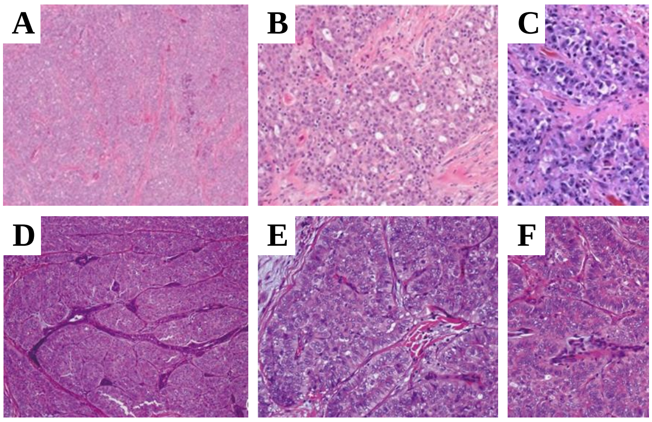

Figure 1. Morphology comparison of mice MGT in two groups via HE staining. A, B, and C were the images of mice MGTs in the CG obtained by HE staining under 40×, 100×, and 400×microscopic observation. D, E, and F were the images of mice MGTs in the TG obtained by HE staining under 40×, 100×, and 400×microscopic observation.

PE of FasL and Bcl-2 Detected by Immunohistochemistry

FasL and Bcl-2 were both located in the cell membrane and cytoplasm in the immunohistochemical staining of mice BC tissues, as shown in figure 2. The expression level of FasL protein in the MGTs of the CG was greatly lower than that of the TG. Meanwhile, the proportion of positive cells in the MGTs of the two groups, which was the PR, was calculated and analyzed, and the results were shown in figure 3. The PR of FasL PE was 20.25% in the CG and 89.65% in the TG, there was a statistically obvious difference (P<0.01). The expression rate of Bcl-2 protein in the MGTs of the TG was greatly higher than that of the CG, and the PR of FasL PE in the MGTs of the TG and CG were 87.96% and 31.48%, respectively, and there was a highly obvious difference (P<0.01).

Analysis of mRNA Expression Levels of FasL and Bcl-2

The expression levels of mRNA of FasL and Bcl-2 in the MGTs of mice in the TG and CG were analyzed by qPCR, and the results were shown in figure 4. The expression level of FasL mRNA in the MGTs of mice in the TG was 5.82±4.37, while that of mice in the CG was 1.27±0.12, and there was a statistically obvious difference (P<0.05). The expression level of Bcl-2 mRNA in the MGTs of mice in the TG was 18.97±2.65, while that in the MGTs of mice in the CG was 2.02±0.54, and there was an extremely obvious difference (P<0.01).

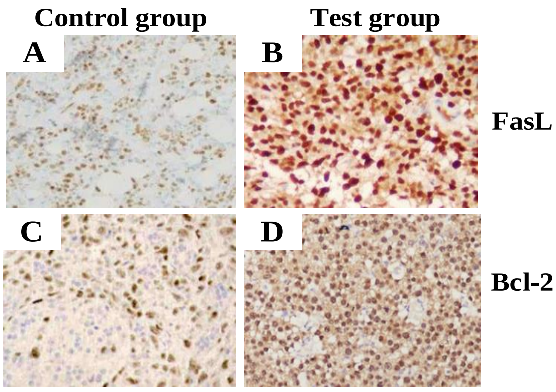

Figure 2. Comparison of PE of FasL and Bcl-2 in MGT detected by immunohistochemistry. A. FasL expression of CG. B. FasL expression of TG. C. Bcl-2 expression of CG. D. Bcl-2 expression of TG.

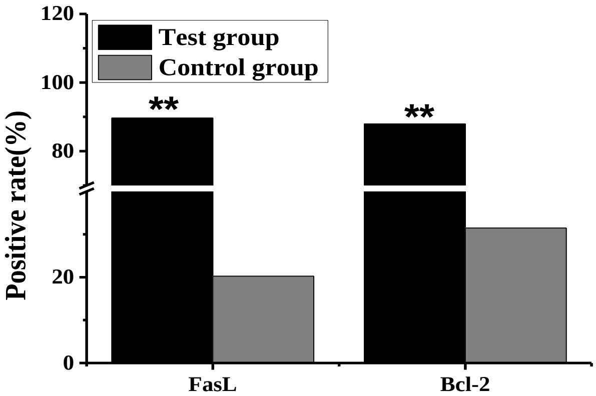

Figure 3. Comparison of PR of MGT of the two groups. **indicated there was a statistically obvious difference compared with the CG, P<0.01.

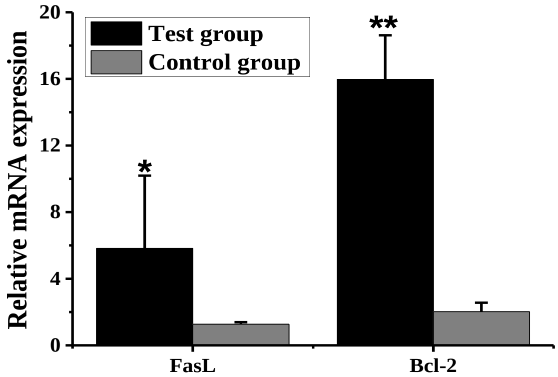

Figure 4. Comparison of mRNA expression levels of FasL and Bcl-2 in MGT.*indicated there was a statistically obvious difference compared with the CG, P<0.05. **indicated there was a statistically obvious difference compared with the CG, P<0.01.

Analysis of BC Cell Apoptosis Detection Results

Breast tissue sections of the two groups of mice were examined by the TUNEL method to detect the apoptosis of mice breast cells, and the results were shown in figure 5. Almost none of the mammary gland cells of the CG mice were stained by TUNEL, indicating that there was no apoptosis in the CG cells. Almost all the mammary gland cells of the TG mice were stained by TUNEL, indicating that the mammary gland cells in the TG entered apoptosis state. And the apoptosis rates of cells in the MGTs of the two groups of mice were calculated and analyzed, the results were shown in figure 6. The apoptosis rate of mice mammary gland cells in the TG was (19.79±3.53) %, and that of the CG was (2.93±0.28) %, and there was an extremely obvious difference (P<0.01).

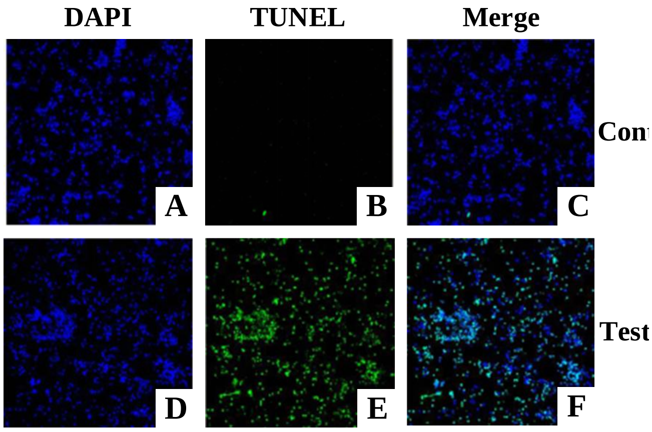

Figure 5. Analysis of BC cell apoptosis detection results.

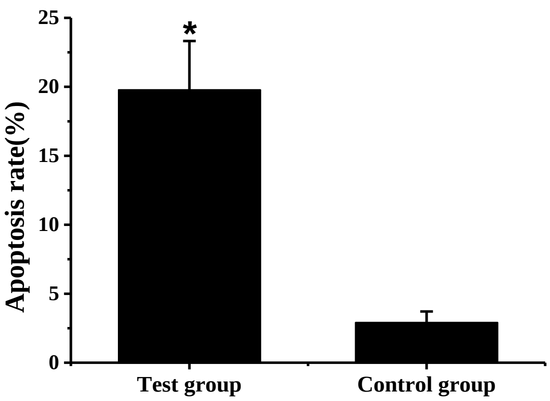

Figure 6. Comparison of apoptosis rate of the two groups.* indicated there was a statistically obvious difference compared with the CG, P<0.01.

Apoptosis and proliferation of tumor cells play an important role in the carcinogenesis and development of tumors. Studies showed that Fas and ligand FasL were important regulating factors inducing apoptosis and maintaining the body's balance (19,20). The effect of DMAD on FasL and Bcl-2 expression level and breast cell apoptosis were studied by inducing BC in mice of the TG through DMBA. The results showed that the expression level of FasL protein in the MGTs of the CG was greatly lower than that of the TG, and the PR of FasL expression detected by the immunohistochemical method was 20.25%, which was statistically significant compared with the PR of 89.65% in the TG (P<0.01). The relative expression of FasL was further analyzed by qPCR, and it was found that the mRNA expression of FasL in the MGTs of mice in the TG was 5.82±4.37, which was greatly different from that in the CG 1.27±0.12 (P<0.05). At present, a large number of studies showed that (21,22), the PR of FasL in BC tissues was greatly higher than that in normal MGT. And studies have found that FasL expression level was greatly different in different MGTs, with the highest expression level in BC tissues, followed by breast tumors, both of which were greatly higher than that in normal MGTs (23). Fan et al. (2020) (24) also found that in the immunohistochemistry results, the expression rate of FasL positive cells increased in normal cells, mammary gland hyperplasia, and mammary gland. The results of this study also showed that FasL expression in BC tissues was higher than that in normal MGT, which was consistent with the current research results. This indicated that the expression of FasL had a certain correlation with the tumor progression of BC. TUNEL method was used to detect the apoptosis of mice breast cells, it was found that almost none of the cells in the CG was stained by TUNEL, while almost all the cells in the TG were stained by TUNEL. Cubero et al. (2019) (25) found that in the immunohistochemical detection of MGT, a large number of cells in FasL positive MGT showed apoptosis, indicating that FasL expression was high, and a large number of cells in MGT showed apoptosis compared with the normal CG. The results of this study were consistent with those of Cubero et al.

Bcl-2 gene, also known as apoptosis suppressor gene, is currently recognized as a tumor cell apoptosis suppressor (26). Bcl-2 can induce apoptosis through a variety of pathways and mechanisms, and is involved in the carcinogenesis and development of tumors

(27,28). In this study, the expression rate of Bcl-2 protein in the MGTs of the two groups of mice was analyzed, and it was found that the positive expression rate of Bcl-2 protein in the TG was 87.96%, which was greatly higher than that in the CG (31.48%), and there was an extremely obvious difference between the two groups (P<0.01). The expression level of Bcl-2 mRNA was analyzed by qPCR, and it was found that the expression level of Bcl-2 mRNA in the MGTs of mice in the TG was 18.97±2.65, while that in the MGTs of mice in the CG was 2.02±0.54, and there was an obvious significant difference (P<0.01). The results of Zhou et al. (2019) (29) showed that the PR of Bcl-2 in MGT was 28.42%, and there was no statistically obvious difference from the control normal MGT, which was contrary to the results of this study. The possible reason was that Bcl-2 had both anti-proliferation and anti-apoptosis effects in cells, and was expressed in normal MGT, which could regulate the normal tissue to survive rather than apoptosis (30). Studies showed that with the cell proliferation, the anti-proliferation effect of Bcl-2 gradually weakened, showing the anti-apoptotic activity, so the expression level of Bcl-2 in the early stage of the tumor was not greatly changed. It was also possible that the expression of Bcl-2 was related to the degree of tumor cell proliferation and the size of tumor cells (31), resulting in different research results. The results of this study showed that the apoptosis rate of mammary gland cells in the TG was (19.79±3.53) %, which was greatly higher than that in the CG (2.93±0.28) %, and there was an extremely obvious difference (P<0.01). The study results of Senturk et al. (2020) (32) also showed that the apoptosis rate in BC tissues was greatly higher than that in normal tissues, which was consistent with the results of this study. The apoptosis rate was increased and the apoptosis was active. Meanwhile, the proliferation rate was accelerated and the DNA replication error rate was increased due to the rapid proliferation, which further increased the probability of normal cells turning into tumor cells. After the carcinogenesis of precancerous lesions, the apoptosis mechanism was inhibited, but the cells still proliferated at a fast speed, promoting the formation of tumors (33, 34).

Based on the above research results, DMBA upregulated FasL and Bcl-2 expression levels in tissues and cells, inhibited apoptosis of BC cells, resulting in BC. There were still some deficiencies in this study. The changes of DMBA on FasL and Bcl-2 expression were analyzed, yet the effects of different does of DMBA on FasL and Bcl-2 expression were not studied. In the following work, the relationship between DMBA dose and FasL and Bcl-2 expression and apoptosis of BC cells will be studied. In conclusion, DMBA inhibited the apoptosis of BC cells by regulating the up-regulation of FasL and Bcl-2 expression.

Acknowledgments

None.

Conflict interest

The authors declare no conflict of interest.

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