Original Article

Combinatorial effect of zoledronic acid and irradiation on the prevention of DMBA-induced precancerogenic changes in the mammary tissues of rats

ABSTRACT

Background: At present, the rates of breast cancer are continuously increasing, with over a million new cases being diagnosed worldwide each year. Hence, the development of new breast cancer chemopreventive drugs with acceptable efficacy and toxicity that are suitable for use for a protracted period of time is urgently needed. The present study investigated the potential preventive effects of zoledronic acid [ZOL] and radiotherapy [RT], both alone and in combination, on precancerogenic changes on the breast tissues of females.

Materials and Methods: Wistar rats were treated with 7,12-dimethylbenz [a] anthracene [DMBA] at the acute phase. Fifty female rats were divided into seven groups: Control group [I]; ZOL, group [II]; RT, group [III]; DMBA, group [IV]; DMBA + RT, group [V]; DMBA + ZOL, group [VI]; and DMBA + ZOL + RT, group [VII].

Results: The treatment of DMBA-exposed rats with ZOL and RT, both alone and in combination, successfully upregulates the transcriptional levels of Bax, caspase-3, caspase-9, p21, and BRCA 1 in mammary tissues, which may account for the elevated apoptotic activities observed and the eventual inhibition of tumor growth. The administration of RT and ZOL both alone and in combination was found to be effective for inhibiting the DMBA-induced precancerogenic changes on breast tissues and modulating the expression of apoptosis-associated proteins in the acute phase.

Conclusions: The combination of RT and ZOL was more effective than either agent alone. Our results suggest that the administration of ZOL and irradiation in combination can offer maximal protection against DMBA-induced mammary precancerogenic changes.

KEY WORDS: 7,12-dimethylbenz[a] anthracene, breast cancer, zoledronic acid radiotherapy

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INTRODUCTION

The most common type of cancer among women is breast cancer.[1] Breast cancer is associated with excessive cell proliferation, dysregulation of cellular differentiation, and insufficient apoptosis.[2] Some genes, including BRCA1, BRCA2, p21 and Bcl-2, have been linked to breast cancer susceptibility and development.[3] Women with risk factors are encouraged to perform routine breast cancer surveillance for early detection, and high-risk women may take preventative action, including surgical prophylaxis and preventive therapy with medicine.[4] Thus, recognition of the limitations of current diagnostic, surgical, and therapeutic approaches for breast cancer has resulted in a new focus on procedures for the early detection and treatment of breast cancer.

DMBA, a well-known polycyclic aromatic hydrocarbon, is a widespread genotoxic and tumorigenic environmental pollutant.[5] DMBA-induced rat mammary tumor is an important preclinical animal model of breast cancer.[6] This tumor induced by this model is morphologically and histologically similar to that observed in human estrogen-dependent breast cancer. The rat mammary tumors induced by DMBA express many biochemical and molecular markers, such as p53, BRCA, Bcl2 and p21, which are also expressed in human mammary tumors.[6] The process of carcinogenesis impairs apoptosis, which results in development of the malignant phenotype.

Genetic factors, including mutations in BRCA 1 and BRCA 2, contribute to 10% of breast cancer incidence in the western world.[7] Although the past several decades have seen substantial improvement in the outcome of breast cancer, primarily due to its early diagnosis and the increased use of hormonal and adjuvant chemotherapies, preventive approaches using novel agents may be considered

the winning strategy for reducing the morbidity and mortality of breast cancer.[8,9] A recently published consensus statement significantly underscores the value of preventive therapy for limiting the devastating impact of breast cancer.[10] Two selective estrogen receptor modulators, namely tamoxifen and raloxifene, are currently approved drugs by the United States Food and Drug Administration (US-FDA) for the prevention of breast cancer in women with high risk for the development of this disease.[10,11] Nevertheless, concern with serious side effects precludes the widespread and long-term use of these medical options.[12] Hence, the development of new breast cancer chemopreventive drugs with acceptable efficacy and toxicity that are suitable for use for a protracted period of time is urgently needed.[12]

Zoledronic acid [ZOL], which is clinically applied to treat cancer-induced bone diseases, appears to possess direct anti-tumor activity through apoptosis induction by inhibiting mevalonate pathway and prenylation of intracellular small G proteins.[13] At the same time prophylactic low-dose whole breast radiotherapy may be an alternative to surgery in the high-risk patients.

To this end, the present study was conducted to determine whether the combination of ZOL and RT exerts a protective effect by stimulating apoptosis during DMBA-induced mammary precancerogenesis using the expression of the apoptosis-associated proteins BRCA1, P21, Bcl-2, Bax, and caspases 3 and 9 as markers in the acute period.

MATERIALS AND METHODS

Animals

Female Wistar rats [200 ± 4.5 g] aged 55 days were obtained from the Research Laboratory of Experimental Animals at University of İnönü and housed in a room maintained at constant temperature [22 ± 2°C] and humidity [55 ± 5%] with 12 h of light and 12 h of darkness each day. The rats were acclimated to the environment for one week prior to the initiation of the experiment. All of the procedures involving rats were conducted in strict compliance with relevant laws, the Animal Welfare Act, the Public Health Services Policy, and the guidelines established by the Institutional Animal Care and Use Committee of the University.

Experimental design

The rats were divided according to body weights [BW], which were similar, into seven equal groups of seven animals each. Group 1 [control] received the normal diet and served as a negative control, and all of the other groups received the same diet as the control group and the following treatments: ZOL, group [II]; RT, group [III]; DMBA, group [IV]; DMBA + RT [V]; DMBA + ZOL, group [VI]; and DMBA + ZOL + RT, group [VII].

ZOL [Novartis Pharma Stein AG] was applied at a dose of 7 μg/kg via the intraperitoneal (i.p). route, and DMBA [Sigma Chemical Co., St. Louis, MO, USA] at a dose of 20 mg/kg was administered via the i.p. route. Treatment was continued for 13 days. In RT groups all of the rats were anesthetized i.p. with 20–30 mg/kg ketamine hydrochloride [Rotex, Germany] prior to irradiation. Each rat was then restained and taped by the tail and legs on an acryl plate at a supine position. Six Gy RT was applied on the fourteenth day as total body irradiation with Co 60 device.

Animals were observed daily, and all the necessary data were recorded. The experiment was terminated at the end of the 15 days. All animals were sacrified by cervical dislocation after an overnight fast. Blood was collected and normal, and suspicious lesions were rapidly removed, measured, and rinsed in physiological saline.

Western blot analysis

The blood samples were centrifuged at 3,000 × g for 10 min. The consisting serum was separated and stored at -80°C until further analysis. Samples of mammary tissues from the animals of each group were collected for western blot analysis. The specimens were used fresh or stored at -80°C. Frozen tissue samples were homogenized in phosphate-buffered saline [PBS] with protease inhibitor cocktail [Calbiochem, San Diego, CA, USA], and the protein concentration was analyzed. The samples [20 mg of protein per lane] were mixed with sample buffer, boiled for 5 min, separated by SDS-polyacrylamide [12%] gel electrophoresis under denaturing conditions, and electroblotted onto nitrocellulose membranes. The nitrocellulose blots were washed in PBS and blocked with 1% bovine serum albumin in PBS for 1 h prior to application of the primary antibody. The primary antibody was diluted [1:1,000] in the same buffer containing 0.05% Tween-20. The nitrocellulose membrane was incubated overnight at 4°C with protein antibody. Antibodies against Bcl-2, Bax, cleaved caspases 3 and 9, and ß-actin were purchased from Santa Cruz [Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horse-radish peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific binding was detected using diaminobenzidine and H_2O_2 as the substrates. The blots were performed at least four times to confirm the reproducibility of the data. The protein levels were analyzed densitometrically using an image analysis system [Image J; National Institute of Health, Bethesda, MD, USA].[6]

Histopathological analysis

Breast tissue samples for histological evaluation were prepared in 10% buffered formalin and then embedded in paraffin. The sections were stained with hematoxylin and eosin [HE]. Fresh tissues were used for each experimental process. The breast tissues were evaluated histopathologically through a blinded review.

Statistical analysis

Data are described median as minimum and maximum values, Kruskal-Wallis test was used for group comparisons.

Kruskal-Wallis test after multiple comparisons were made by the method of Conover. 0.01 and 0.05 level of significance for all tests was considered.

RESULTS

The effects of ZOL and RT on the expression of Bcl-2, Bax, caspase-3, and caspase-9 in the mammary tissues are shown in Figure 1. The administration of DMBA significantly elevated Bcl-2 expression and significantly decreased the Bax, caspase-3, caspase-9, P21, and BRCA 1 expression levels in comparison to the control animals. However, the administration of ZOL or the application of radiotherapy alone or in combination to DMBA-treated animals significantly decreased Bcl-2 expression and significantly increased the Bax, caspase-3, and caspase-9, P21, and BRCA expression levels compared with the levels observed in the controls.

In the histomorphological sections, various criteria, including ductal epithelial hyperplasia, dysplasia, apoptosis were evaluated. All of the these morphological changes was observed in the group treated with DMBA [Figure 2]. Severity of histopathological changes due to DMBA decreased in ZOL, RT and combination of ZOL + RT groups [Table 1].

DISCUSSION

The present study evaluated the potential preventive effects of ZOL and RT administered alone and in combination on the precancerogenic changes observed in rats treated with DMBA through an analysis of the expression levels of markers associated with apoptosis during the acute phase. These results provide the first demonstration of the potent anti-precancerogenic activity of these agents.

In the present study, the treatment of DMBA-exposed rats with ZOL and RT successfully upregulated the transcriptional levels of Bax, caspase-3, and caspase-9 and downregulated Bcl-2 expressionin mammary tissues, which may account for the observed elevated apoptotic activities and the eventual inhibition of precancerogenic changes.

The Bcl-2 family regulates mitochondrial membrane permeabilization and therefore controls the release of apoptotic factors from the mitochondrial intermembrane space that are overexpressed in approximately 80% of breast malignancies.[14] These factors initiate the caspase cascade. Active caspase-3 is capable of cleaving and activating procaspase-8 and thereby amplifying and speeding up the apoptotic process.[15] Bcl-2 proteins can be either pro-apoptotic [e.g. Bax] or anti-apoptotic [e.g. Bcl-2 proper 9].[16] This is a primary factor when considering the efficacy of cancer chemopreventive applications. Yin *et al.* reported that Bax plays a direct role on tumor suppressionin a mouse epithelial tumor model.[17] In addition, Chen *et al.* indicated that the caspase family plays an important role in apoptosis.[18]

Table 1: Histopathologically value as mean±SD in all the groups

	Hyperplasia	Dysplasia	Apoptosis
Control	0	0	0
ZOL	0	0	0
RT	0.02±0.0	0.3	1
DMBA	0.3±0.2[a]	0.7±0.5[a]	1.7±0.4[a]
DMBA+RT	0.05±0.02[b]	1±O[c]	1.2±0.4[b]
DMBA+ZOL	0[b]	1.5±0.5[ab]	1.3±0.8[c]
DMBA+RT+ZOL	0[c]	1.12±0.3[b]	1.4±0.5

Data points with superscripts a (compared to control), b (compared to DMBA), c (compared to DMBA+RT), d (compared to DMBA+ZOL), indicate significant difference at the level of $P<0.05$. SD=Standard deviation, ZOL=Zoledronic acid, RT=Radiotherapy, DMBA=Dimethylbenz [a] anthracene

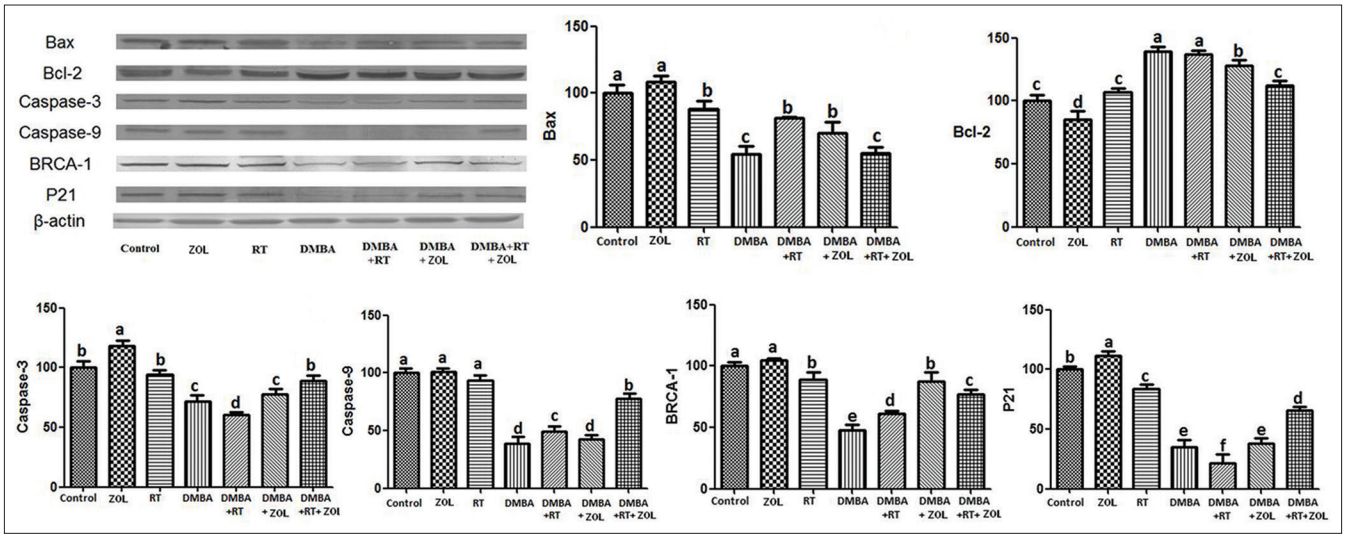

Figure 1: Levels of Bax, Bcl-2, caspase-3, caspase-9, BRCA-1 and P21 expression, as determined through western blot strips [Panel A]. Effect of ZOL and RT on the Bax [Panel B], Bcl-2 [Panel C], caspase-3 [Panel D], caspase-9 [Panel E], BRCA-1 [Panel F] and P21 [Panel G] protein expression levels in breast tissues from DMBA-induced rats

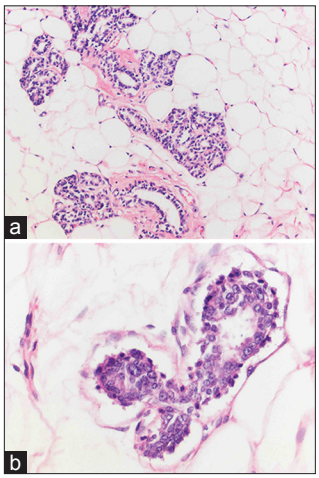

Figure 2: (a) Control breast tissue [HE200]. (b) Dysplasia was observed in the group treated with DMBA [HE400]

The present study revealed that ZOL and RT treatment resulted in increased expression of p21. Through a western blot analysis, Lan *et al*. showed that the inhibitory effect of ZOL on carcinogenesis is related to the extent of inhibition of phosphorylated-mammalian target of rapamycin [p-mTOR].[19] It is known that mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, and protein synthesis and transcription and triggers the production of p21, whereas inhibition of mTOR is known to be related to this effect.[20,21]

We also found that the combination of RT and ZOL exhibit increased effects compared with each agent alone. Koto *et al*. showed that ZOL increased the degree of radiation-dependent apoptosis.[22] In addition, Kijima *et al*. previously reported that ZOL clinically potentiates the antitumor effects of RT on bone metastases from renal cell carcinoma [RCC].[23] Their study clearly demonstrated that 10 μM ZOL exerts a radiosensitizing effect directly on RCC cells by enhancing RT-induced caspase-3 activation through a molecular mechanism. ZOL has been considered a radiosensitizer *in vitro* and has been reported to enhanceradiosensitization in various malignancies, including esophageal squamous cancer, fibrosarcoma, and RCC.[22-24]

We also found the ZOL treatment resulted in increased expression of BRCA-1. BRCA-1 is also known as a tumor suppressor gene, is related to carcinogenesis and is a potential molecular target for the induction of apoptosis.[25] In sporadic breast tumors, which are observed in the majority of breast cancer cases, epigenetic mechanisms contribute to its reduced expression in the absence of mutations in the BRCA-1 gene.[26] In addition, in the present study, irradiated breast tissue exhibited high levels of the BRCA-1 gene. Chiang *et al*. reported that the irradiated breast tissue exhibited very low progenitor cell activity *in vitro*.[27] Given the emerging evidence that BRCA-1 tumors originate from luminal progenitor cells, their observations suggest that the preferential and long-lasting elimination of the luminal ductal epithelium may partly underlie the mechanism of RT-associated reduction in the recurrence of BRCA-1-associated cancer.[27] RT is associated with a reduced recurrence of sporadical and BRCA-1-associated breast cancer.[27]

Infact, whole-breast low-dose RT has been proposed as an alternative to prophylactic mastectomy. The potential sensitivity of luminal epithelial cells to RT may provide a means to eradicate critical cells of gene-associated tumors, the exploitation of which may guide the development of novel preventive options for this select patient population. ZOL may be useful in conjunction with radiotherapy for patients with genetic risk factors.

ZOL treatment in combination with RT may prevent DMBA-induced precancerogenic changes, which may be associated with reduced tumors and the upregulation of tumor suppressor genes in breast cancer. RT markedly reduces the luminal compartment and diminishes progenitor cell activity. Our observation is compatible with the concept that radiation reduces the number of luminal progenitor cells in gene-associated tumors, thus leading to reduced cancer incidence.

Both treatment with ZOL alone and treatment with RT alone reduced breast tumor development, but the combination treatment showed stronger activity [Figure 1]. These results provide the first demonstration that the combination of ZOL and RT exerts potent anti-precancerogenic changes in a DMBA-induced rat mammary cancer model. Further studies may be needed to illuminate the effect of the combination therapy on cancer treatment from different perspectives.

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