Original Article

Effects of genistein and daidzein, in combination, on the expression pattern of biomolecular markers (p53, PCNA, VEGF, iNOS, Bcl-2, and Bax) during 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis in Sprague-Dawley rats.

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ABSTRACT

Evaluation of molecular markers in the mammary tissues has prognostic and therapeutic values. Aim of the present study was to evaluate the effects of genistein and daidzein, in combination, on the expression pattern of biomolecular markers such as p53, PCNA, VEGF, iNOS, Bcl-2, and Bax during DMBA induced mammary carcinogenesis in Sprague-Dawley rats. Mammary tissues were routinely processed and paraffin embedded, 2-3µm sections were cut in a rotary microtome and mounted on clean glass slides. Immunohistochemical staining of mammary tissue was performed and each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively and scoring was made. Immunohistochemical analysis revealed over expression of p53, PCNA, VEGF, iNOS, Bcl-2 and decreased expression of Bax in the mammary tissues of DMBA treated rats. Oral administration of genistein+daidzein (20mg+20mg/kg bw) in combination, to DMBA treated rats significantly down regulated the expression of p53, PCNA, VEGF, iNOS, Bcl-2 and up regulated the expression of Bax proteins. Our results suggest that the pleiotropic and synergistic effects of genistein and daidzein might have reduced cellular proliferation, decreased angiogenesis and induced apoptosis during DMBA induced mammary carcinogenesis in Sprague-Dawley rats.

1. Introduction

Breast cancer is one of the commonest cancer affecting 1.3 million women worldwide each year. It is the second leading cause of cancer death in women today and about 465,000 die annually due to this cancer. The incidence of breast cancer is rapidly rising in both developed and developing countries of the world. It has been reported that breast cancer afflicts one in eight women in the USA during their lifetime and USA has the highest annual incidence rates of breast cancer in the world [1]. The incidence of breast cancer is increasing at alarming pace in India, mainly in metropolitan cities, where one in 22 women is likely to suffer from breast cancer during their life time. Also, breast cancer is becoming the number one cancer in females pushing the cervical cancer to second place [2]. 7,12-dimethylbenz(a)anthracene (DMBA), a potent site and organ specific carcinogen, is mostly used to induce mammary carcinomas in Sprague-Dawley rats since the tumor developed in the mammary glands of rats closely mimics human breast cancer and share several morphological and molecular similarities [3].

Breast cancer occurs as the result of a progressive accumulation of genetic aberrations in the cells of mammary gland and it can be curable if diagnosed at an early stage. Detection of biochemical or molecular markers in tumors could have repercussion on prognosis and monitoring. Investigations of tumor cell proliferative activity, invasive capacity, angiogenesis, and apoptosis could help to detect tumor stage as well as therapeutic response [4]. Currently, molecular markers such as p53, PCNA, VEGF, iNOS, Bcl-2, and Bax are used routinely as prognostic and predictive markers for breast cancer.
Molecular targeted agents are currently being studied in all treatment settings including chemoprevention, which deals with the inhibition, prevention or reversal of carcinogenesis using natural or synthetic entities [5, 6]. Genistein and daidzein, prominent isoflavones of soy beans, have been associated with reduced risk of breast cancer [7]. Studies have demonstrated that prepubertal exposure to genistein decreased the risk of mammary tumorigenesis in female rats [8, 9]. Similarly, daidzein significantly inhibited DMBA-induced mammary tumors in rats [10]. However, reports also suggested that genistein increased proliferation of cells in tumors and increased the weight of estrogen dependent mammary adenocarcinomas in rat models [11, 12]. The protective effect of these phytoestrogens on mammary cancer therefore remains inconclusive [13]. In order to resolve the controversies, genistein and daidzein still continue to be the target agent for breast cancer chemoprevention studies. Previous study from our laboratory demonstrated the chemopreventive potential of genistein and daidzein, in combination, during DMBA induced mammary carcinogenesis in Sprague-Dawley rats [14]. The present study attempts to evaluate the effects of genistein and daidzein, in combination, on the expression pattern and inter-correlation of biomolecular markers such as p53, PCNA, VEGF, iNOS, Bcl-2, and Bax during DMBA induced mammary carcinogenesis in Sprague-Dawley rats.

2. Materials and Methods

2.1. Rats

Forty female Sprague-Dawley rats, six weeks old, were obtained from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The rats were housed in polypropylene cages at room temperature (27 ± 2°C) with relative humidity 55 ± 5%, in an experimental room. In Annamalainagar, the LD (light: dark) cycle is almost 12:12h. The rats were provided with standard pellet diet (Purchased from Amrut Laboratory Animal Feed, Mysore Feeds Limited, Bangalore, India) and water ad libitum. The experimental design (Proposal No. 578 dated. 25.07.2008) was approved by the Annamalai University animal ethical committee (Register number 160/1999/CPCSEA), Annamalainagar. The rats were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use.

2.2. Chemicals

Genistein and daidzein were purchased from Shaanxi Sciphar Biotechnology Co. Ltd, China. DMBA and Dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Other chemicals and solvents used were of analar grade.

2.3. Experimental Design

Forty rats were divided into four groups and each group contained ten rats. Group 1 rats received the excipient (single dose of 1 ml of emulsion of sunflower oil and physiological saline, s.c.) and 1 ml of 2% DMSO (p.o) throughout the experimental period served as vehicle treated control. Rats in groups 2 and 3 were induced mammary carcinogenesis by providing single subcutaneous injection of 25 mg of DMBA in 1ml emulsion of sunflower oil (0.75ml) and physiological saline (0.25ml) [15]. Group 2 rats received no other treatment. Group 3 rats were orally administered with genistein+daidzein (20mg+20mg/kg body weight, dissolved in 1 ml of 2% DMSO) starting one week before the exposure of the carcinogen and continued till the experimental period. Group 4 rats were orally administered with genistein+daidzein (20mg+20mg/kg body weight, each dissolved in 1 ml of 2% DMSO) alone throughout the study. The experiment was terminated at 16th week and all rats were sacrificed by cervical dislocation. The mammary tissues were harvested and preserved in 10% buffered formalin used for immunohistochemical studies.

2.4. Immunohistochemical staining

Mammary tissues were routinely processed and paraffin embedded. 2-3μm sections were cut in a rotary microtome and mounted on clean poly lysine coated glass slides, dried at 37°C. Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H2O2 in methanol for 10 min. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D.H2O; 0.37 g EDTA/L D.H2O; 0.2 g trypsin, pH 6.0) for 10 min, followed by washing step with tris-buffered saline (8 g NaCl; 0.605 g Tris, pH 7.6). The tissue section was then incubated with power BlockTM reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent for 15 min at room temperature to block non-specific binding sites. The tissue sections were then incubated with the respective primary antibody (DAKO p53, PCNA, VEGF, iNOS, Bcl-2, and Bax) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 min at room temperature. After rinsing with tris-buffered saline, the antigen-antibody complex was detected using 3,3′-diaminobenzidine, the substrate of horseradish peroxidase. When acceptable colour intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a resinous mounting medium.

Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. The percentage of positive cells were scored according to the method of Lyzogubov et al [16] as follows: 3+ = strong staining, more than 50% of cells were stained; 2+ = moderate staining, between 20 and 50% of cells were stained; 1+ = week staining, between 1 and 20% of cells were stained; 0 = negative, less than 1% of cell staining.

3. Results

The immunoreactivity pattern and intensity of positively stained cells of p53, PCNA, VEGF, iNOS, Bcl-2, and Bax in control and experimental rats in each group are shown in figures 1 (a-d), 6 (a-d) and Table 1 respectively.

The analysis showed a positive staining for p53, PCNA, VEGF, iNOS, Bcl-2 and Bax in tumor tissues, which is more pronounced as compared to normal tissues. It was observed 60%, 85%, 55%, 40%, 55% and 30% positive staining for p53, PCNA, VEGF, iNOS, Bcl-2 and Bax respectively in tumor tissues (group 2). We observed nuclear expression of p53 and PCNA, cytoplasmic expression of VEGF, iNOS, Bcl-2 and Bax in the mammary tissues of DMBA alone treated rats.

It was found that p53, VEGF, iNOS, Bcl-2 and Bax were negatively stained in the mammary tissues of control rats. In normal mammary epithelium, lower expression of PCNA however observed. Oral administration of genistein+daidzein (group 3) to DMBA treated rats significantly down regulated the expression of p53, PCNA, VEGF, iNOS, Bcl-2, and up regulated the expression of Bax proteins. Rats treated with genistein+daidzein alone showed no significant difference in the expression pattern of p53, VEGF, iNOS, Bcl-2 and Bax proteins as compared to control rats (group 1).
Fig. 1 depicts immunoeexpression of p53 in (a) control rats (no expression), (b) DMBA alone treated rats (over-expression), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (no expression).

Fig. 2 depicts immunoeexpression of PCNA in (a) control rats (Weak expression), (b) DMBA alone treated rats (up regulated), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (Weak expression).
Fig: 3 depicts immunoexpression of VEGF in (a) control rats (no expression), (b) DMBA alone treated rats (up regulated), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (no expression).
Fig. 4 depicts immunoexpression of iNOS in (a) control rats (no expression), (b) DMBA alone treated rats (up regulated), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (no expression).

Fig. 5 depicts immunoexpression of Bcl2 in (a) control rats (expression not detectable), (b) DMBA alone treated rats (up regulated), (c) DMBA+ genistein and daidzein treated rats (down regulated) and (d) Genistein and daidzein alone treated rats (expression not detectable).
Fig. 6 depicts immunoeexpression of Bax in (a) control rats (no expression), (b) DMBA alone treated rats (down regulated), (c) DMBA + genistein and daidzein treated rats (up regulated) and (d) Genistein and daidzein alone treated rats (no expression).
Table 1 shows the score of positively stained cells of p53, PCNA, VEGF, iNOS, Bcl-2, and Bax in control and experimental rats in each group.

<table>
<thead>
<tr>
<th>Groups/Markers</th>
<th>P53</th>
<th>PCNA</th>
<th>VEGF</th>
<th>iNOS</th>
<th>Bcl-2</th>
<th>Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0+</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>0+</td>
<td>1+</td>
</tr>
<tr>
<td>Control (Vehicle)</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>DMBA+ Genistein Daidzein</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Genistein Daidzein alone</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are given as number of rats (n = 10). The percentage positive cells were scored as: 3+ = strong staining, more than 50% of cells were stained, 2+ = moderate staining, between 20 and 50% of cells were stained, 1+ = week staining, between 1 and 20% of cells were stained, 0 = negative, less than 1% of cell staining.

4. Discussion

Tumor cells grow and multiply rapidly by adopting host cell survival pathways and thus agents which protect these pathways from carcinogenic agents have great potential for the treatment of cancer. The possible mechanisms so far reported for the anticancer effect of natural products include protection of tissues from carcinogenic stimuli, suppression of procarcinogenic regulatory mechanisms and cell proliferation, modulation of inter cell communication signals, destruction or removal of tumor cells, induction of apoptosis and suppression of angiogenesis [17].

p53, a nuclear phosphoprotein, regulates cell-cycle at the G1/S phase and apoptosis in response to DNA damage. The wild type p53 is a short half-life protein and potent tumor suppressor. However, cancer-associated mutant forms of p53 have long half-life that promotes tumorigenesis and tumor aggressiveness [18]. A diverse multitude of cellular stress, such as DNA damage, hypoxia, mitogens, etc. activates mutant form of p53. Mutant p53 gene and p53 protein expression has been reported in 3050% of mammary carcinogenesis [19]. Maru et al [20] reported the over expression (50%) of mutant p53 in breast carcinoma of women age 30 years and younger. Wild type p53 also down regulates Bcl-2 and up regulates Bax after exposure to DNA damaging agents [21].

Apoptosis, the programmed cell death, maintains a balance between cell death and cell renewal by removing excess, damaged or abnormal cells. Bcl-2 family is a 25-kd integral membrane proteins that govern mitochondrial outer membrane permeabilization and can be either anti-apoptotic (Bcl-2) or pro-apoptotic (Bax) proteins. It has been reported that Bcl-2 prevents Bax/Bak oligomerization, which in turn lead to the release of several apoptogenic molecules from the mitochondria [22]. Bax/Bcl-2 ratio determines the fate of a cell in response to stress and measurement of the expression of these proteins together may provide valuable predictive information about outcome of the cancer treatment. Abrogation of Bcl-2/Bcl-xL expression as well as reinforcement of Bax expression not only cause tumor regression but also render them more sensitive to apoptosis-inducing treatment. Immunooexpression pattern of Bcl2 and Bax has been well documented in almost all types of cancer including breast cancer [23].

Proliferating Cell Nuclear Antigen (PCNA), a highly conserved 36-kd nuclear protein of DNA polymerase-delta, has been found to be an useful marker to assess tumor cell proliferation and progression [24]. Alterations in gene that regulate the timing of the events in the cell cycle contribute to carcinogenesis. Over-expression of PCNA was reported in various malignancies including breast cancer [25]. The tumor with high index of PCNA had more aggressive growth and recurrence, resulting in low survival rates [26].

Tumor angiogenesis actually starts when tumor cells send signals to surrounding normal host tissue to make proteins such as VEGF. Both VEGF and iNOS can induce angiogenesis in tumor cells. VEGF expression in the cytoplasm of tumor cells has been reported in breast cancer tumor specimens [27]. Excessive and prolonged iNOS mediated NO generation has been linked with inflammation and tumorigenesis. A positive correlation between iNOS expression and angiogenesis has been reported [28].

Soy isoflavones play an important role in reducing the incidence of breast and prostate cancers in Asian countries. Genistein and daidzein exerted anticarcinogenic potential through estrogenic/antiestrogenic activity, induction of cell cycle arrest, inhibition of oxidative stress, and activation of cell death signaling [29, 30, 31, 32]. Isoflavones in combination have shown pronounced inhibitory effects on breast cancer cell proliferation [33]. It has been demonstrated that oral administration of genistein and daidzein, in combination, significantly protected tumor formation (80%) in DMBA induced mammary carcinogenesis in Sprague-Dawley rats [14].

Genistein, an inhibitor of protein tyrosine kinases has been reported to inhibit the growth of several cancer cells through the modulation of genes that are related to the process of cell growth or signal transduction pathways [34]. Genistein induced apoptosis, G2 phase arrest of cell cycle and inhibited cell proliferation in a variety of human cancer cell lines, regardless of p53 status [35]. Daidzein was shown to alter cell cycle distribution in HeLa cells [36]. Genistein and daidzein modulated IGFI-induced in vitro proliferation and apoptotic resistance in the cell line via inhibition of multiple intracellular signaling pathways involving tyrosine kinase activity [37]. Genistein induced apoptosis was tested in all cancer cells including breast cancer cells MDA-MB-231, MDA-MB-435, and MCF-7 by means of caspase-3 activation and down-regulation of Bcl-2, Bcl-xL, and HER-2/neu [38, 30]. Kazi et al [39] reported that genistein induced apoptosis through the activation of Bax. Bax/Bcl-2 ratio was increased in MCF7 breast cancer cells after 24 h of genistein treatment, [40]. Genistein was the most potent inhibitor of angiogenesis in vitro and in vivo among the isoflavone compounds [41].
5. Conclusion

In the present study, oral administration of genistein+daidzein, in combination, to DMBA treated rats significantly down regulated the expression of p53, PCNA, VEGF, iNOS and Bcl-2 whereas up regulated the expression of Bax in the mammary gland. The present study thus demonstrated that the pleiotropic and synergistic effects of genistein and daidzein in combination might have reduced cellular proliferation, decreased angiogenesis and induced apoptosis during DMBA induced mammary carcinomaogenesis in Sprague-Dawley rats.

6. References


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6. References