Chemoprevention of radiation-induced mammary tumors in rats by bezafibrate administered together with diethylstilbestrol as a promoter

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Pregnant Wistar-MS strain rats were irradiated with 2.6 Gy of γ-rays at day 20 of pregnancy. Rats in the control group (n = 48) were then implanted with a diethylstilbestrol (DES) pellet at 35 days after weaning, while being fed a control (MB-1) diet. The incidence of mammary tumors was 89.6% within 1 year. In the experimental group (n = 22), a bezafibrate (0.15%) diet was initiated immediately after weaning, and 35 days after weaning a DES pellet was implanted. Administration of dietary bezafibrate together with DES-implantation continued for a period of 1 year, at which time the experiment was terminated. The incidence (27.3%) of the mammary tumors in the bezafibrate-fed rats was less than one-third of that in the control rats. Compared with the control group, the number of mammary tumors per tumor-bearing rat in the bezafibrate-treated group was reduced. For clarification of the mechanism of the chemopreventive effects of bezafibrate, lipid and hormone concentrations in serum were measured. Bezafibrate-fed rats showed a significant decrease in serum prolactin (56%) and triglyceride (63%) concentrations, and a significant increase in serum estradiol-17β (3.8-fold), cholesterol ester (2.0-fold) and TSH (2.0-fold) concentrations in comparison with the control rats. The bezafibrate diet inhibited the formation of DES-induced pituitary tumors. However, the development of mammary glands in the bezafibrate-fed rats was stimulated more than that in the control rats treated with DES alone. The present results demonstrate that bezafibrate is effective in preventing mammary tumors induced by radiation together with DES, possibly by reducing prolactin and triglyceride concentrations.

Introduction

Results from epidemiological studies have shown that the dietary intake of fatty acids might influence the risk of breast cancer (1,2). A positive association between dietary fatty acids and tumor growth has also been identified in animal models of mammary carcinogenesis (3). In comparison with animals fed a low-fat diet, female rats fed high-fat diets during the promotional stage developed significantly more N-nitrosomethylurea (NMU)-induced mammary adenocarcinomas (4,5). Bezafibrate (Figure 1) is an analogue of clofibrate that is currently used in the clinical treatment of hyperlipidemia. The treatment of rats with bezafibrate led to a marked hypolipidemic effect, which was reflected in the reduction of plasma cholesterol and triglyceride levels (6,7). Previous studies in our laboratory (8,9) and others (10,11) demonstrated that diethylstilbestrol (DES) promotes the development of radiation-induced mammary tumors in rats. The present study was designed to evaluate the chemopreventive activity of bezafibrate against DES-dependent promotion/progression of rat mammary tumors initiated by γ-rays. In order to elucidate the mechanism of its anti-tumor action, the effects of bezafibrate on endocrine systems, such as the pituitary and ovarian functions, were determined.

Materials and methods

Materials

\[\text{[2,4,6,7-^3\text{H}]}\text{Estradiol-17\beta} \text{ (specific activity, } 4 \text{ TBq/mmol)} \text{ and } [\text{17\alpha-methyl-}^3\text{H}](\text{7a-methyl-17-propionylestra-4,9-dien-3-one} \text{ (R5020) (specific activity, } 3 \text{ TBq/mmol)} \text{ were purchased from Du Pont/NEN Research Products (Boston, MA). Estradiol-6-[\text{10-carboxymethyl-oximino-2,12\text{jodohistamine}}] \text{ (specific activity, } 74 \text{ TBq/mmol) was obtained from Amersham (Aylesbury, UK). Bezafibrate was kindly supplied by Kissei Pharmaceutical Co. (Matsumoto, Japan). DES was obtained from Sigma (St Louis, MO). Pellets were prepared in a medical grade Silastic tube (Dow Corning Co., Midland, MI) and were filled with } 3 \text{ mg of DES mixed with } 27 \text{ mg of cholesterol. The diet containing } 0.2\% \text{(w/w)} \text{ bezafibrate was prepared in biscuit form by Funabashi Farm (Chiba, Japan) and was sterilized by } \gamma\text{-rays (10 kGy). For the determination of the change of the drug concentration by radioisotopes, bezafibrate in the sterilized diet was analyzed by high-performance liquid chromatography (HPLC) after extraction (12) and was estimated as } 0.15\% \text{ (w/w) } \text{A basal diet (MB-1) of the same form was used for the control experiments. The major components of MB-1 were as follows: total carbohydrate, } 54.2\%; \text{ proteins, } 24.5\%; \text{ fat, } 4.0\%; \text{ fibers, } 3.8\%; \text{ moisture, } 7.7\%; \text{ ash, } 5.8\%; \text{ their concentrations did not change by the sterilization.}

Animals and treatment

The rats used in the present study were treated and handled according to the recommendations for the protection of laboratory animals. Female Wistar-MS rats, bred in this Institute, were kept at 23 ± 1°C in a controlled environment (14-h light-10-h dark). They received water and food ad libitum. Seventy pregnant rats received whole-body irradiation with 2.6 Gy γ-rays (0.17 Gy/min) from a 137Cs source at day 20 of pregnancy (the presence of a vaginal plug denoting day 1) and were divided into two groups after weaning. For the control experiment, 48...
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![Cumulative Incidence](image)

**Fig. 2.** Cumulative incidence of the development of mammary tumors in irradiated rats treated with diethylstilbestrol (DES) and fed a control diet or bezafibrate diet.

<table>
<thead>
<tr>
<th>Table I. Mammary tumor prevention by bezafibrate in radiation-initiated and DES-promoted rat tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>No. of rats used</td>
</tr>
<tr>
<td>Rats with tumors (%)</td>
</tr>
<tr>
<td>No. of tumors per tumor-bearing rat</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fibroadenomas</th>
<th>Adenocarcinomas</th>
<th>Iball's index</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tumors</td>
<td>47</td>
<td>27</td>
</tr>
<tr>
<td>Latency period (months)</td>
<td>8.9 ± 0.4</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Latency period (months)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Iball's index</td>
<td>6 (27.3)</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Table II. Biological effects of long-term treatment with bezafibrate

<table>
<thead>
<tr>
<th>Control diet (n = 5)</th>
<th>Bezafibrate diet (n = 8)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>262 ± 18</td>
<td>254 ± 8</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>12.2 ± 1.0</td>
<td>15.2 ± 1.0</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>545 ± 100</td>
<td>428 ± 33</td>
</tr>
<tr>
<td>Ovaries (mg)</td>
<td>109 ± 19</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>Pituitary (mg)</td>
<td>37 ± 9</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

rats were fed a basal diet and were then implanted with a DES pellet in the interscapular area under light anesthesia by sodium pentobarbital at 35 days after weaning. Serving as the experimental group, 22 rats were fed a diet containing 0.15%(w/w) bezafibrate beginning immediately after weaning, and were implanted with a DES pellet at 35 days after weaning. The pellets were replaced every 8 weeks. The rate of release of DES from the pellet was approx. 0.38 ± 0.01 μg/day (9). The rats were examined for palpable mammary tumors for 1 year starting from the date of pellet implantation. When tumors >2 cm in diameter were detected, the rats were killed by CO2 asphyxiation and the tumors were removed.

**Whole mounts of mammary glands**

After bezafibrate treatment for 1 year, the entire inguinal mammary glands were dissected from the inner surface of the skin, retaining as much of the connective tissue as possible, and spread and dried slightly on filter paper.

![Whole mount preparations](image)

**Fig. 3.** Whole mount preparations of mammary glands. (a) Diethylstilbestrol (DES)-treated rats fed the control diet, (b) DES-treated rats fed a diet containing 0.15% bezafibrate. The scale bar on each panel corresponds to 1 mm.

![Hormone concentrations](image)

**Fig. 4.** Hormone concentrations in the serum. Open bar, control diet (n = 5); closed bar, 0.15% bezafibrate diet (n = 5). Mean ± SE. Significant difference from the control, *\(P < 0.01\); **\(P < 0.05\). Pg, progesterone; \(E_2\), estradiol-17β; DOC, deoxycorticosterone; DHEA-S, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone.

![Histological examination](image)

**Histological examination**

The mammary tumors were fixed immediately in 10% formalin buffered with 0.1 M phosphate buffer (pH 7.2) and deparaffin in acetone, the preparations were stained with alun carmine, destained in ethanol and stored in cedar oil (13).

After fixing in 10% formalin buffered with 0.1 M phosphate buffer (pH 7.2), each paraffin section (4 μm in thickness) was prepared and stained with hematoxylin and eosin (H&E). The tumors were classified as adenocarcinoma or fibroadenoma according to the criteria for the classification of rat mammary tumors (14).
Chemoprevention of mammary tumors by bezafibrate

**Fig. 5.** Cholesterol and triglyceride concentrations in the serum. Open bar, control diet (n = 5); closed bar, 0.15% bezafibrate diet (n = 6); Mean ± SE. Significant difference from the control: *P < 0.01; **P < 0.05; CE, cholesterol ester; FC, free cholesterol; TG, triglyceride.

**Fig. 6.** Immunohistochemical localization of prolactin in the pituitary glands of diethylstilbestrol (DES)-treated rats fed the control diet (a, b) or bezafibrate diet (c, d). (a, c) Staining with non-immunized rabbit sera, (b, d), staining with anti-prolactin rabbit antiserum. The scale bar on each panel corresponds to 100 μm.

**Fig. 7.** Concentrations of estrogen receptors and progesterone receptors in mammary tumors. Left, estrogen receptors; and right, progesterone receptors. Open circles represent data for fibroadenoma, and closed circles denote those for adenocarcinoma. MBS: maximum binding sites.

**Assay of steroid receptors**

The tumor tissues (1 g) were homogenized in 10 mM Tris–HCl buffer (pH 7.4) containing 1.5 mM EDTA-Na and 1 mM dithiothreitol. Homogenates were centrifuged at 105,000 g for 1 h at 4°C, and the obtained cytosol fraction was used for assay of the receptors. Estrogen receptor (ER) and progesterone receptor (PgR) in the cytosol fraction were analyzed by a dextran-coated charcoal method using [2,4,6,7-^3^H]estradiol-17β and [17a-methyl-^3^H]R5020, respectively, as radioligands (15,16). Maximum binding sites and dissociation constant (Kd) values for the receptors were determined by a Scatchard plot analysis (17).

**Immunohistochemical detection of prolactin in pituitary glands**

The pituitary glands were fixed in Bouin's solution without acetic acid for 4 h. The tissues were dehydrated and embedded in paraffin. Sections (4 μm in thickness) of the pituitary glands were deparaffinized, and immunohistochemical staining was performed by streptavidin–biotin methods (Histofine, SAB-PO(R9) kit, Nichirei Co., Tokyo) using anti-rat prolactin S-9 anti-serum (NIDDK) supplied by the National Hormone and Pituitary Program (Baltimore, MD). Immunoreactive tissues were visualized by horse radish peroxidase using 3,3'-diaminobenzidine. The specificity of staining was confirmed by the use of non-immunized normal rabbit serum. The sections were counterstained lightly with hematoxylin.

**Radioimmunoassay of hormones**

A blood sample collected from each rat by cardiocentesis under Nembutal (sodium pentobarbital, Abbott Lab. North Chicago, IL) anesthesia was allowed to clot and was then centrifuged to obtain serum. The sera were frozen immediately and stored at −80°C until the assay was started. Concentrations of prolactin, LH, FSH and TSH in each serum sample were determined with an NIDDK radioimmunoassay kit (National Hormone and Pituitary Program). Estradiol-17β was measured by a modification of the method of Watanabe et al. (18). The serum concentrations of progesterone, deoxycorticosterone, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA–S), cholesterol and triglyceride were assayed by commercially available kits. Sensitivities were 0.2 ng/ml for LH, FSH, TSH, prolactin and progesterone, 0.1 ng/ml for DHEA, 20 ng/ml for DHEA–S, 0.02 ng/ml for DOC, 1 pg/ml for estradiol-17β, and 2 mg/dl for cholesterol and triglyceride.

**Iball's index and statistical analysis**

Iball's index was calculated as follows: the ratio of incidence (%) to the average latency period in days was multiplied by 100 (19). All statistical analyses were performed using StatView-J4.5 software (Abakus Concepts, Inc., Berkeley, CA). Statistical analyses were conducted by χ^2 test and Log-rank test for tumor
incidence, by Student's t-test for the level of significance of differences between pairs of mean values for body and organ weights and hormone concentrations and by Mann–Whitney U test for the receptor concentrations. The significance level was set at \( P < 0.05 \).

Results

Effects of dietary bezafibrate on development of mammary tumors

When the control rats received whole body irradiation with 2.6 Gy γ-rays at day 20 of pregnancy and then were treated with DES after weaning, a high incidence (89.6%) of tumorigenesis of the mammary glands was observed. The tumor incidence (27.3%) in the bezafibrate-fed rats was less than one-third (\( P < 0.0001 \)) of that in the control rats (Table I). The Iball’s index for overall development of mammary tumors in the bezafibrate-fed rats was also one-third of that in the control group. The number of mammary tumors per tumor-bearing rat in the bezafibrate-treated group (1.2 ± 0.2) was 63% (NS, \( P = 0.224 \)) of that in the rats fed the control diet (2.0 ± 0.2). The latency period was 9.7 ± 0.5 months in the control group and 9.6 ± 1.5 months in the bezafibrate-fed group for appearance of adenocarcinoma, and 8.9 ± 0.4 months in the control group and 8.8 ± 0.7 months in the bezafibrate-fed group for fibroadenoma. No significant difference of the latency period was observed between the groups. As shown in Figure 2, the administration of dietary bezafibrate together with DES implantation in the irradiated rats significantly decreased the cumulative incidence of mammary tumors for the 1-year period, compared with the control diet group. The appearance of first palpable tumors was delayed by approximately 5 months in the bezafibrate-fed group compared with that in the control group.

Biological changes by long-term administration of bezafibrate

The body and organ weights at the terminus of the experiments are summarized in Table II. A significant reduction of body weight was observed in all rats receiving DES implantation. When the diet containing 0.15% bezafibrate was administered for 1 year, the body weight of the treated rats was comparable to that of the rats fed the control diet (\( P = 0.640 \)). Liver weight was increased slightly (1.2-fold) by the administration of bezafibrate, but no significant difference was observed (\( P = 0.077 \)). No hepatic tumor had developed in the rats of the bezafibrate-treated group. The weights of uterus and ovaries in the bezafibrate-fed rats decreased to about 80% of those in the control rats, but no significant difference between the two groups was observed (\( P = 0.213 \) for uterus and 0.212 for ovaries). Rats in the control group treated with DES alone had pituitary tumors (37 ± 9 mg). Several of the pituitary tumors were seen macroscopically to have friable hemorrhagic tumors, and by microscopical observation they exhibited adenoma with marked vascularization. Bezafibrate thus had a potent preventive activity against the formation of DES-induced pituitary tumors in the irradiated rats.

Whole mounts of mammary glands

Whole mounts of the mammary glands of the irradiated rats were prepared at the termination of the experiment in order to examine the development of the glands by treatment with DES alone or both DES and bezafibrate. Alveolar buds with branched lactiferous ducts were observed in all areas of the mammary glands of the rats implanted with DES for 1 year (Figure 3a). In contrast, the development of lobuloalveolar structures was shown in the mammary glands of all the irradiated rats treated with DES together with bezafibrate (Figure 3b).

Serum concentration of hormones and lipids

The serum concentrations of ovarian, adrenal and pituitary hormones were assayed 1 year after the start of the administration of dietary bezafibrate with DES implantation in the irradiated rats. The serum estradiol-17β and progesterone concentrations in the rats fed the bezafibrate diet were 3.8- and 1.2-fold higher than those in the control rats, respectively (Figure 4). The increased level of estradiol-17β was significantly different (\( P < 0.01 \)) from the value of the control, but that of progesterone was not (\( P = 0.618 \)). Compared with the control rats, the bezafibrate-fed rats showed a 50% decrease (\( P < 0.05 \)) in serum prolactin concentration, but a 2.0-fold increase (\( P < 0.01 \)) in TSH. No significant differences in deoxycorticosterone (\( P = 0.660 \)), DHEA–S (\( P = 0.335 \)), LH (\( P = 0.219 \)), and FSH (\( P = 0.066 \)) were observed between the two groups. The concentration of triglyceride was reduced in the bezafibrate group (\( P < 0.05 \)) (Figure 5), but the concentration of cholesterol ester was increased (\( P < 0.01 \)). The DHEA concentrations obtained were 0.8 ± 0.2 ng/ml in the control group and 0.3 ± 0.06 ng/ml in the bezafibrate-fed group (\( P < 0.05 \)).

Prolactin in pituitary glands

As shown in Figure 6a, prolactin was detected in numerous pituitary tumor cells of DES-treated rats fed the control diet. Bezafibrate completely inhibited the formation of DES-induced pituitary tumors, and also caused the reduction of prolactin cell number and of the pituitary prolactin level elevated by DES (Figure 6b).

**Table III. Estrogen receptor and progesterone receptor incidence in mammary tumors**

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of tumors tested</th>
<th>ER(+)PgR(+)</th>
<th>ER(+)PgR(-)</th>
<th>ER(-)PgR(+)</th>
<th>ER(-)PgR(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33</td>
<td>22</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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ER and PgR in mammary tumors
Table III shows the results of the Scatchard plot analysis of ER and PgR in the cytosol fraction obtained from the homogenates of the mammary tumors >2 cm in diameter. Many (66.7%) of the mammary tumors that developed in the rats fed the control diet were of the ER(+)PgR(+) type, and only one fibroadenoma was ER(-)PgR(-). Conversely, all tumors in the bezafibrate-fed rats were ER(+), and one adenocarcinoma and one fibroadenoma had no detectable PgR levels. The maximum binding sites (52.4 ±21.3 fmol/mg protein, \( n = 5 \)) for ER and PgR in the mammary tumors >2 cm in diameter were 1.3-fold higher than those (40.5 ±17.2 fmol/mg protein, \( n = 5 \)) of the control tumors. The concentrations of ER in the fibroadenomas were 9.6 ± 2.1 fmol/mg protein, and those of PgR were 15.6 ± 5.8 fmol/mg protein. The maximum binding sites for ER and PgR in the fibroadenomas were 52.4 ±21.3 fmol/mg protein, \( n = 5 \) and 15.6 ± 5.8 fmol/mg protein, \( n = 5 \), respectively.
fmol/mg protein (n = 20) in the controls and 11.8 ± 2.4 fmol/mg protein (n = 5) in the bezafibrate-fed rats (no significant difference, P = 0.533). No statistical comparison was conducted of the maximum binding sites of ER and PgR in adenoacarcinomas that developed in both groups, because there were only two adenocarcinomas in the bezafibrate-fed rats.

Discussion

This is the first report in the literature showing that bezafibrate is effective in cancer prevention. To date, bezafibrate had only been shown to modify tumor radiosensitivity by reduction of hemoglobin/oxygen affinity by reduction of hemoglobin/oxygen affinity (20). The results of the present study clearly document the ability of dietary bezafibrate to inhibit DES-dependent promotion and progression of radiation-induced mammary carcinogenesis. The long-term administration of bezafibrate blocked the formation of DES-induced pituitary tumors. The prolactin concentration of the serum was significantly reduced by bezafibrate treatment in spite of the increase in the serum estradiol-17β level. The significant suppression of serum prolactin concentrations in the bezafibrate-treated rats strongly suggested that the mechanism of the chemoprevention by bezafibrate in rats is an alteration in prolactin-induced mammary tumorigenesis. In previous studies of chemoprevention of radiation-induced mammary tumors by our laboratory (21), similar observations of the hormone concentration and the decrease of the prolactin level under an increased concentration of estradiol-17β were seen by administration of dietary DHEA together with DES implantation. Interestingly, bezafibrate significantly enhanced the growth rate of MAC 16 tumor cells in vivo, but not in vitro (22). The stimulatory action of bezafibrate correlated with the plasma level of free fatty acids arising from the catabolism of adipose tissue (22). The level of dietary fat intake has been shown to influence the development of DMBA-induced mammary tumors (23). The stimulation of fatty acid elongation or desaturation activities by bezafibrate may be reflected by the decrease in plasma triglycerides (6). The same authors (6) also reported an increase in Δ6-desaturase activity by bezafibrate, suggesting a decrease in linoleic acid content in lipoproteins. During bezafibrate treatment, the concentration of linoleic acid was significantly reduced and the γ-linolenic acid content was significantly higher (24). An increase of linoleic acid intake cause an enhancement in DMBA-induced rat mammary tumors (25,26), and a stimulation of growth rates in a human breast cancer cell line, MDA-MB-231 (27), and transplantable mammary adenocarcinoma (28,29). In contrast, oleic acid (5,27), docosahexaenoic acid (27) and arachidonic acid (30) each exhibited a dose-related inhibition of the growth rate of mammary tumors. The mechanism of modification in mammary tumorigenesis by the change of fatty acid metabolism is not well established at present.

Stahlberg et al. (31) have reported that a significant reduction of HMG-CoA reductase activity was observed in rats fed a 0.1% bezafibrate diet. HMG-CoA reductase catalyzed a rate-limiting reaction for biosynthesis of farnesyl-pyrophosphate and cholesterol. Therefore, inhibition of HMG-CoA reductase activity reduced the amounts of farnesyl-pyrophosphate available for farnesylation of ras proteins. However, the serum concentration of cholesterol was not reduced by long-term administration of bezafibrate in the present study, suggesting no reduced activity of HMG-CoA reductase. We doubt that one of the mechanisms in the chemoprevention of mammary tumors by long-term treatment with bezafibrate is suppression of the farnesylation of ras proteins.

In conclusion, the results obtained in the present study demonstrated that bezafibrate, an antilipidemic drug, was effective in preventing mammary tumors induced by radiation together with DES. Bezafibrate reduced the levels of prolactin and triglyceride in serum, but further research is needed to establish its mechanism of modification in mammary tumorigenesis by the change of fatty acid metabolism.

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