Phytoestrogen-Rich Diets Modulate Expression of Brca1 and Brca2 Tumor Suppressor Genes in Mammary Glands of Female Wistar Rats

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ABSTRACT

Phytoestrogens are natural compounds with anticancer, proliferation, differentiation, and chemopreventive effects, for which several mechanisms have been proposed. In the present study, modulation of Brca1 and Brca2 expression by different phytoestrogen-rich diets has been investigated in ovariectomized Wistar rats. Two hundred mammary glands were harvested in three independent experiments. Brca1 and Brca2 mRNAs were quantified by real-time quantitative reverse transcription-PCR, and their proteins by immunohistochemistry. The first experiment compared the influence of different phytoestrogens [flax-seed, isoflavones (IFs), or rutin]. A 10% increase in Brca1 mRNA expression was shown after flax-seed consumption, whereas no variation was noted for Brca2 mRNA, nor for Brca1 and Brca2 proteins. In the second experiment, two soy IFs sources (Novasoy or Soylife) were given at different concentrations to the animals. Only Brca2 mRNA was increased and only at high doses. Finally, the effect of IFs was compared with that of estradiol. An increase in mRNA for both genes was noted after estradiol treatment and with the highest dose of IFs.

In conclusion, our results show that IFs, given in the diet at different doses, are able to increase Brca1 and Brca2 mRNA in ovariectomized female Wistar rat. However, no variation in Brca1 or Brca2 protein expression was demonstrated, whatever the experimental conditions were.

INTRODUCTION

There is a large amount of epidemiological and in vitro data suggesting that phytoestrogens may prevent carcinogenesis and inhibit tumor growth in breast cancer, as well as prevent osteoporosis (1–3) and cardiovascular disease (4).

Phytoestrogens belong to a broad group of biologically active compounds that have attracted attention recently. Those molecules occur widely in edible plants, and share with estradiol a phenolic ring and a pair of hydroxyl groups. The major classes of current interest from a nutritional and health perspective are the IFs5 and the lignanes, although other polyphenols such as quercetin may exhibit light estrogenic activities.

Two major tumor suppressor genes of human breast cancer have been described, BRCA1 and BRCA2 (5, 6). In sporadic human breast cancers, although somatic mutations have not been detected (7), alterations in BRCA1 and BRCA2 mRNA level have been observed (8).

The rat Brca1 gene has been map precisely to chromosome 10 (9), and its sequence is highly homologous to that of mice (88%) and humans (81%). Brca1 mRNA is expressed in most tissues, with highest levels in testis, consistent with human and mouse data (5, 10). Brca1 mRNA levels are also high in ovary, spleen, lung, and liver, and lower in mammary gland, kidney, heart, and brain, and undetectable in skeletal muscle (9).

Rat Brca2 was cloned and mapped to chromosome 12 by Yamada et al. (11) in 1997 and shares a large homology with human BRCA2 (12). Expression studies demonstrate an 11–12 kb transcript with tissue-specific patterns of expression consistent with human BRCA2.

Studies of BRCA1 and BRCA2 function strongly suggested that these genes play critical roles in the regulation of mammalian epithelial cell growth (13). Variations in Brca1 and Brca2 expression were also observed in adult mouse tissues during mammary gland development associated with pregnancy (14, 15).

Previous in vitro studies demonstrated modulation of BRCA1 and BRCA2 expression6 by genistein but not daidzein.7 This is why the present study aimed to investigate the effect of other phytoestrogens in an OVX rat model.

MATERIALS AND METHODS

Animals and Diets. These studies were conducted in accordance with current legislation on animal experiments in France. Female Wistar rats (n = 190; 195 days old) were purchased from I.N.R.A. (Clermont-Ferrand Theix, France). All of the rats were housed individually at 21°C with a 12-h light/dark cycle in metal cages. Throughout the experimental period the rats had free access to water and were fed daily a humidified (1 ml water/g food) soy protein-free semipurified diet (I.N.R.A., Jouy-en-Josas, France; Table 1).

First Experiment: Comparison of Different Phytoestrogen-Rich Diets (Fig. 1A). Ninety-day-old Wistar female rats were either SH (n = 10) or OVX (n = 30). On the day after surgery (day 0), OVX animals were randomly allocated to three groups of 10 rats each and given the standard diet supplemented with either 0.5% soy IFs, which corresponded to a dose of 16 μg/g body weight per day (Soylife, Giessen, the Netherlands), 0.25% rutin (a quercetine, glycoside; Sigma, France), or 10% flax-seeds (providing ~0.2% lignans; Biofar, France) for 90 days. Then, the animals were sacrificed by cardiac punctation.

Second Experiment: Comparison of Two Soy IF Sources (Fig. 1B). In the same way, Wistar female rats were either SH (n = 10) or OVX (n = 60). At day 0, the OVX animals were randomly allocated to six groups of 10 rats each and given either Novasoy [A, Novasoy IF compound 152–400; Archer Daniels Midland Co., Decatur, IL], containing 338 mg/g as total IFs (genistein 159 mg, daidzein 156 mg, glycitein 33 mg, i.e. a genistein-daidzein:total IF ratio of 90%)] or Soylife [S, Soylife 100, batch n° 32K/154/H, containing 35 mg/g as total IFs (5.55 mg genistin, 15.36 mg daidzin, and 14.09 mg glycitein, i.e. a genistein-daidzin:total IFs ratio of 60%); Soylife] at three concentrations for each products: 20 (A1), 40 (A2), and 80 (A3), or 4 (S1), 8 (S2), and 16 (S3) μg/g body weight/day for 90 days. The six diets were prepared by mixing powdered soybean-IF concentrate with the semipurified diet.

Third Experiment: Comparison of IFs and Estradiol (Fig. 1C). Wistar female rats were either SH (n = 10) or OVX (n = 50). Ninety days after

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6 To whom requests for reprints should be addressed, at Laboratoire d’Oncologie Moléculaire, Centre Jean Perrin, 38 rue Montalembert, BP 392, 69011 Clermont-Ferrand, cedex 01, France. Phone: 33-4-73-27-80-50; Fax: 33-4-73-27-80-42; E-mail: Yves-Jean.Bignon@cjp.fr.

7 The abbreviations used are: IF, isoflavone; SH, sham-operated; OVX, ovariectomized; Ct, threshold cycle.

8 Unpublished observations.
Mammary glands were harvested, then snap-frozen in liquid nitrogen at the time of collection.

**mRNA Extraction.** Mammary glands were weighed quickly, then ground thoroughly with a French press, and the powder disrupted in a potter with the appropriate amount of RTL buffer containing β-mercaptoethanol, according to the weight of the gland. The solution was homogenized by passing lystate at least 10 times through a 30-gauge needle fitted to an RNase-free syringe. Total mRNA isolation was performed using RNA easy mini kit (Qiagen) according to the manufacture’s protocol.

**Real-Time Quantitative PCR.** Total mRNA was reverse transcribed using a Pharmacia kit according to the manufacturer’s conditions. Expression of *Brca1* and *Brca2* mRNAs was assessed by quantitative real-time PCR (system Taqman) by the comparative C_T method (16, 17).

Probes and primers used for the quantification were chosen with the help of Primer Express and are summarized in Table 2.

Single-stage PCR was carried out in a final volume of 25 μl containing 5 μl of the Reverse Transcription (RT) reaction mix (diluted to 25 ng) and 12.5 μl of Taqman Universal PCR Master mix, which containing 200 nM of each primer pair and probe (*Brca1* or *Brca2*), 200 μM dNTP, 400 μM dUTP, 5 mM MgCl_2, 1.25 units of Ampli Taq Gold, 0.5 units of amperase uracil-glycosylase, and 50 nM of 18S rRNA primers and probe.

The PCR procedure consisted of 2 min at 50°C, 94°C for 10 min for the uracil-N glycosylase activation, 50 cycles with a initial denaturation of 15 s at 95°C, and finally an extension step of 1 min at 60°C, using an ABI prism 7700 sequence detection system (Applied Biosystems, ZA Courtabeuf, France).

The relative quantification of *Brca1* and *Brca2* expression was performed using the comparative C_T method (18), which consists of the normalization of the C_T of the target gene (*Brca1* or *Brca2*) to the C_T of an endogenous reference gene (i.e. 18S rRNA; ΔC_T = C_T target gene - C_T 18S rRNA). C_T for *Brca1* or *Brca2* of each SH rat mammary gland were quantified, and the C_T average was determined with the other mammary gland tested (ΔΔC_T = ΔC_T sham-operated − ΔC_T target gene).

**Acetone Fixation of Frozen Mammary Glands and Immunohistochemistry.** Cryostat sections (5 μm) were cut at −40°C. After 1 h stabilization, they were air-dried for 30 min then fixed in acetone at room temperature for 10 min and air-dried 10 min. Sections were washed in PBS and incubated for 45 min with antibodies against *Brca1* or *Brca2* (Table 3). After washing in PBS, the slides were incubated for 30 min with biotinylated goat antirabbit or rabbit antigoat immunoglobulins from Dako LSAB 2 system Peroxidase (Dako

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**Table 1 Composition of the soybean-protein-free powdered semipurified diet**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (g/kg)</th>
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</thead>
<tbody>
<tr>
<td>Casein</td>
<td>180</td>
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<tr>
<td>Sucrose</td>
<td>210</td>
</tr>
<tr>
<td>Maize starch</td>
<td>430</td>
</tr>
<tr>
<td>Cellulose</td>
<td>100</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>25</td>
</tr>
<tr>
<td>Rapeseed oil</td>
<td>25</td>
</tr>
<tr>
<td>Vitamin mixture (with cholecalciferol 32.25 µg/kg)</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mixture [with (g/kg) Ca 2.3, P 1.6, Mg 0.42]</td>
<td>18.5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>1.5</td>
</tr>
</tbody>
</table>

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**Fig. 1.** A, schematic representation of the three experimental protocol for animals and diets. B, S and A corresponded respectively to the Soylife and the Novasoy. The rats were fed with Novasoy at three different doses (A1 = 20, A2 = 40, or A3 = 80 μg/g body weight) and with Soylife at three different doses (S1 = 4, S2 = 8, or S3 = 16 μg/g body weight). The rats received three different doses (IF20 = 20, IF40 = 40, or IF80 = 80 μg/g body weight).

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**Fig. 2.** Quantification of (A) *Brca1* and (B) *Brca2* mRNA by real time quantitative RT-PCR of mammary glands from SH or OVX rats after treatment for three months with Soylife (IF (0.5%)), flax-seed (lin; 10%) or rutin (0.25%). (A) *Brca1* mRNA. (B) *Brca2* mRNA.
After a wash in PBS, the slides were incubated for 20 min with streptavidin conjugated to horseradish peroxidase in PBS, before washing again with PBS for 10 min. The slides were then placed in 3-amino-9-ethylcarbazole with acetate buffer for 10 min, in the dark, washed in PBS, counterstained with Mayer's hematoxylin, and mounted in glycerin. All of the incubations and stainings were performed at room temperature. Negative control without primary antibody was performed on all of the cases (Fig. 5).

RESULTS

Quantification of Brca1 and Brca2 mRNA After Treatment with Phytoestrogens. In the first experiment (Fig. 2), the Brca1 mRNA level in SH rats is defined as 1. As observed by Rajan et al. (19), Brca1 mRNA decreased in OVX rats. The flax-seed-rich diet induced a 3-fold increase of Brca1 mRNA, whereas the other diets did not elicit any difference compared with OVX values. Therefore, estrogen deficiency-induced changes in expression were not corrected by IFs or rutine. In contrast, none of the diets restored the basal levels of Brca2 mRNA observed in SH rats.

The second experiment (Fig. 3) investigated the influence of the quality and dose of soy in relation to IF content on Brca1 and Brca2 expression in OVX rats. Neither Soylife nor Novasoy prevented the reduction in Brca1 mRNA expression, compared with SH rats. Both diets provided the same amount of total IFs, whereas the ratio genistein:daidzein was different. In contrast, ovariectomy i.e. 80 mg IF increased the level of Brca2 mRNA by comparison to SH rats.

In the last experiment (Fig. 4), which studied the potential improvement of ovariectomy-induced conditions by phytoestrogens in comparison with estradiol, the two highest doses of IFs (IF40 and IF80) Fig. 3. Effects of Novasoy at three different doses (A1 = 20, A2 = 40, or A3 = 80 µg/g) or Soylife at three different doses (S1 = 4, S2 = 8, or S3 = 16 µg/g) on (A) Brca1 or (B) Brca2 mRNAs by comparison with untreated rats (SH). The quantification was performed with real time quantitative RT-PCR. A, Brca1 mRNA. B, Brca2 mRNA.

Corporation, Carpinteria, CA). After a wash in PBS, the slides were incubated for 20 min with streptavidin conjugated to horseradish peroxidase in PBS, before washing again with PBS for 10 min. The slides were then placed in 3-amino-9-ethylcarbazole with acetate buffer for 10 min, in the dark, washed in PBS, counterstained with Mayer’s hematoxylin, and mounted in glycerin. All of the incubations and stainings were performed at room temperature. Negative control without primary antibody was performed on all of the cases (Fig. 5).

Table 3 Anti-Brca1 and anti-Brca2 antibodies

<table>
<thead>
<tr>
<th>Antibody names</th>
<th>Antigenic specificities</th>
<th>Types</th>
<th>Sources</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Brca1 M20</td>
<td>(aa 1793–1812) COOH-terminal</td>
<td>Goat PoAb Santa Cruz Biotechnologies</td>
<td>1/15</td>
<td></td>
</tr>
<tr>
<td>D16 (aa 2–18) NH₂-terminal</td>
<td>GoPoAb Santa Cruz Biotechnologies</td>
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<td></td>
</tr>
<tr>
<td>H100 (aa 1–100) NH₂-terminal</td>
<td>Rabbit PoAb Santa Cruz Biotechnologies</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Brca2 H300</td>
<td>(aa 2520–2819) internal region</td>
<td>Rabbit PoAb Santa Cruz Biotechnologies</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>N19 (aa 20–38) NH2-terminal</td>
<td>Goat PoAb Santa Cruz Biotechnologies</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Sequence of probes and primers for rat mRNA quantification

<table>
<thead>
<tr>
<th>Names</th>
<th>Forward primer</th>
<th>TaqMan Probe</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brca1</td>
<td>5′-399CAGATTTGATGCTGGAGTGGCAAG′; 3′</td>
<td>5′-406CAGATCTCAGTGTTGCTAACTGTGGG′; 3′</td>
<td>5′-491GTITTTG3TTGCTCTCCATAT′-3′</td>
</tr>
<tr>
<td>Brca2</td>
<td>5′-397TTGAGGACCCAGCCCGGCTG′; 3′</td>
<td>5′-397CTTCGTTACCCCGGCTG′; 3′</td>
<td>5′-382CCGGAGAGACAAAGGTGCA′-3′</td>
</tr>
</tbody>
</table>

* The numbers indicate the position of the first nucleotide in cDNA sequence of Brca1 or Brca2 (GenBank accession no. AF038760 for Brca1, and no. U89653 for Brca2).
significantly increased \textit{Brca1} mRNA, as did estradiol, to higher levels than in SH rats. The same trend was seen with the lowest dose (IF20), without reaching significance value. Only IF80 exhibited increased \textit{Brca2} expression.

\textbf{Distribution of Brca1 and Brca2 Proteins.} We performed immunoperoxidase studies with different antibodies raised against \textit{Brca1} or \textit{Brca2} to ascertain whether different diets changed the intensity of staining or localization of these proteins.

With the three antibodies raised against \textit{Brca1} (H100, M2, and D16), staining was localized to galactophoric epithelial ducts and in the secretion. The same pattern was observed for antibodies against \textit{Brca2} (H300 and N19).

In each experiment, no variation in the range of staining or localization of \textit{Brca1} or \textit{Brca2} was elicited by any treatment (castration or diet; Fig. 5).

\textbf{DISCUSSION}

The purpose of this study was to determine whether phytoestrogen-rich diets might modulate \textit{Brca1} and \textit{Brca2} expression \textit{in vivo}.

We demonstrated previously \textit{in vitro} an increase of \textit{BRCA2} mRNA in MDA-MB-231, an estrogen receptor-negative tumor cell line, and in MCF10a, a normal breast cell line after treatment with genistein. An increase of \textit{BRCA1} mRNA was also observed in MCF7, an estrogen receptor-positive breast tumor cell line, after treatment with daidzein, and in MDA-MB-231 after genistein treatment. No variation in protein expression was noted for \textit{BRCA2}.

However, studies of cultured cells allow molecular dissection of pathways operative in a single cell, whereas research in animal models integrates the complexity of an organ and its different cell types with the dynamic hormonal and physiological status of the animal.
The ideal candidate animal should be relatively easy to characterize genetically and should allow both selective and rapid breeding. The rat is an extremely valuable model for studies of breast cancer susceptibility genes because the characteristics of rat and human breast cancer are so similar.

The rat model was chosen over the mouse model because: (a) the rat mammary tumor resembles that of the human in its spectrum of hormonal responsiveness; (b) mammary cancers in the rat, like the human but unlike the mouse, lack a major viral etiology; and (c) tumors in the rat, unlike the mouse, are rapidly induced by chemicals, radiation, or hormone treatment. The disadvantage of the rat model is that its genetic map is much less characterized than that of the mouse.

Phytoestrogens may be important antioxidants (20, 21). They also disrupt the action of DNA topoisomerase II and ribosomal S6 kinase, which could explain their observed effects on cell cycle, differentiation, proliferation, and apoptosis. In addition, genistein is a potent inhibitor of protein tyrosine kinase (22, 23). Rutin is the common flavonoid glycoside in the diet and is hydrolyzed by intestinal flora to produce biologically active aglycone (a sugar-free flavonoid; 24), and this quercetine compound has powerful antioxidant properties. Flaxseed is the precursor of enterolactone and enterodiol, which exhibit estrogenic properties. Urinary excretion of the mammalian lignans enterodiol and enterolactone has been shown to be linked to reduced risk of breast cancer (25, 26). However, Sathyamoorthy et al. (27) have demonstrated that phenolic compounds such as daidzein, genistein, and enterolactone were able to elicit an estrogen-like response, whereas enterodiol was not.

The risk of breast cancer is increased by exposure to high levels of endogenous and exogenous estrogens (28, 29). Soy foods are rich in precursors of the IFs daidzein and genistein, which are heterocyclic endogenous and exogenous estrogens (28, 29). Soy foods are rich in phytoestrogene; (27) the phytoestrogen-rich diets containing various compounds to stimulate Brca1 and Brca2 mRNA that occurs either during early pregnancy or in OVX animals treated with estradiol and progesterone were significantly greater than that observed for Brca2.

We believe the environment of low endogenous estrogen created by ovariectomy causes genistein and daidzein to be a significant source of estrogen and, hence, allows for the compounds to stimulate Brca1 and Brca2 mRNA. This hypothesis is corroborated by the control group, which received α-estradiol. These diets prevented the decrease in expression and even increased Brca1 and Brca2 mRNA to a 2-fold higher level than that of SH.

Human BRCA1 and BRCA2 proteins, like rat Brca1 and Brca2, are regulated during mammary proliferation and gland development (13–15, 32). Nevertheless, in the present study no variation was observed, whatever the dietary phytoestrogen.

In conclusion, the present study demonstrated that dietary IFs cannot compensate both immediately after surgery for reduced Brca1 expression because of the ovariectomy but can restore after a delay for Brca1 expression. Although IFs when given in high doses induced Brca2 expression, so they can both be compensated and restore an ovariecotomy status.

Furthermore, we will also study by microarray cDNA method a panel of genes that acts with Brca1 and Brca2 after treatment with a phytoestrogen-rich diet to determine the mechanism pathway of IFs.

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