Altered Mammary Gland Differentiation and Progesterone Receptor Expression in Rats Fed Soy and Whey Proteins

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There are suspected links between an animal’s diet, differentiation status of a target tissue, and sensitivity to chemically induced cancer. We have demonstrated that rats fed AIN93G diets made with soy protein isolate (SPI) or whey protein hydrolysate (WPH) had a lower incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced adenocarcinoma than rats fed the same diet made with casein (CAS). The current study was conducted to determine the differentiation status of the mammary glands during development. Offspring of rats (n = 5–10/group) were fed diets made with SPI, WPH, or CAS throughout life (beginning on gestation day 4) and were sacrificed on postnatal day (PND) 21, PND 33, PND 50 or on metaestrous between PND 48 and PND 51. There were no significant differences between the numbers of mammary terminal end buds (TEBs) or lobuloalveoli (LOB) between any of the diets groups at PND 21 or PND 33, but at PND 50 there was an 75% decrease in the mean numbers of TEBs/mm² in the SPI- or WPH-fed rats, compared with the CAS-fed rats (p = 0.09 and p = 0.06, respectively). In rats sacrificed in metaestrous, there were no significant differences in the proliferation index (PI) in the TEBs or LOB between any of the diet groups. In metaestrous rats, there were twice as many cells expressing estrogen receptor β (ERβ; ~60%) compared with estrogen receptor α (ERα; ~30%) in the LOB and 1.5 times more ERβ (~60%) compared with estrogen receptor α (ERα, ~40%) in the TEBs. There were no diet-dependent differences in expression of ERα and ERβ. Similarly, there were no differences between the diet groups in progesterone receptor (PR) expressing LOB cells. However, in the TEBs there was a diet-dependent difference in PR positive cells with a 34% increase (p < 0.05) in the SPI-fed rats and a 38% increase (p < 0.05) in the WPH-fed rats compared with the CAS-fed rats. These results show that the type of dietary protein alters the phenotype of mammary epithelia in the TEBs. The SPI- and WPH-dependent changes in mammary differentiation may contribute to the reduced sensitivity to DMBA-induced mammary cancer in rats fed these proteins.

Key Words: rats; soy protein isolate; whey protein hydrolysate; casein; diet; protein; terminal end bud; lobuloalveoli; progesterone receptor; estrogen receptor.

The concept that components of the diet can modulate carcinogenesis is well established. Results from epidemiological studies demonstrate that the Western diet is a major factor associated with the high incidence of “Western diseases,” including the major hormone-dependent cancers such as breast cancer (Rose, 1986; Trowell and Birkitt, 1981). Low breast cancer incidence in Asian women and in Japanese women in Hawaii consuming a traditional Japanese diet has been linked to a high intake of soybean products (Adlercreutz et al., 1991; Chu et al., 1995; Lee et al., 1991; Messina, 1995; Messina and Barnes, 1991; Muir et al., 1987; Nomura et al., 1978; Setchell et al., 1984). The breast cancer preventing effects of soy have been reinforced by studies demonstrating that soybean diets reduced chemically induced mammary gland tumors (Bagnall et al., 1990). Similar results have been reported for radiation-induced mammary tumors (Troll et al., 1980). Experimental studies have also reported that the consumption of bovine whey milk proteins may also afford cancer prevention (Bounous et al., 1991). In addition, dietary whey protein hydrolysate (WPH) and soy protein isolate (SPI) administered throughout development prevent 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancers in adult female Sprague-Dawley rats (Hakkak et al., 2000). The mechanisms by which soy or whey act to reduce cancer incidence are not known, but soy contains high levels of isoflavones such as genistin that can be estrogenic in their aglycone form (genistein), and some studies have reported that genistein can stimulate mammary gland differentiation that is resistant to cancer (Murrill et al., 1996). We are not aware of any reported studies on the effects of whey on mammary gland development.

Mammary gland development in prepubertal mammals is under the regulation of estrogens that stimulate formation of ductal branches that end in highly proliferative terminal end buds (TEBs; Russo and Russo, 1994, 1995). Maximum TEB density in the female rat occurs at PND 21 and decreases steadily as a result of the actions of increased progesterone and estrogens from the onset of puberty. The TEBs differentiate into less proliferative alveolar buds (ABs) and ultimately into the least proliferative lobuloalveoli (LOB; Russo and Russo, 1978a, 1996). Studies have shown that the highest numbers of chemically induced mammary tumors occurs in rats between
PND 40 to PND 60 when there is a high density of TEBs that are rapidly differentiating into ABs and LOB, a process that requires increased cell proliferation (Russo and Russo, 1996). In addition, the incidence of mammary carcinomas is positively correlated with the number of TEBs in the mammary gland of the young virgin rat at the time of carcinogen exposure (Russo and Russo, 1978b). Therefore, increased differentiation from TEBs to ABs and LOB would result in a net decrease in proliferating cells and might decrease the sensitivity to carcinogens.

The objective of these experiments was to investigate the effects of dietary SPI and WPH on the development of the mammary gland. To this end, we have measured the mammary gland area, densities of mammary gland structures, and several markers important in mammary differentiation including: (1) cell proliferation index (PI), (2) estrogen receptor (ERα and ERβ) expression, and (3) progesterone receptor (PR) expression.

MATERIALS AND METHODS

**Animal treatments.** All animals were housed in an AAALAC approved animal facility with a 12-h light cycle and constant humidity. Studies were approved by the Institutional Animal Care and Use Committee.

**Experiment 1.** Time impregnated (gestation day 4; GD 4) female Sprague-Dawley rats were purchased from Harlan Industries (Indianapolis, IN). Rats were randomly assigned to three groups and fed one of three isocaloric semipurified diets made according to the AIN-93G diet formula (Reeves et al., 1993), except that corn oil replaced soy oil and the protein source was either casein (CAS), whey protein hydrolysate (WPH; New Zealand Milk Products, 1993), or soy protein isolate (SPI; a gift from Protein Technologies International Inc., St. Louis, MO). The protein content of each diet was the same, except that corn oil replaced soy oil and the protein source was either CAS, SPI, and WPH, respectively.

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Mammary epithelial cells or in cells expressing either ERα or ERβ. In the TEBs, PR expressing cells were increased by 34% ($p < 0.05$) in the SPI-fed rats and 38% ($p < 0.05$) in the WPH-fed rats compared with the CAS-fed rats. There was no difference in PR expression between diet groups in LOB cells.

**DISCUSSION**

We have previously reported that in female rats orally gavaged with DMBA at PND 50, there was a significant reduction in mammary tumors in SPI- or WPH-fed rats, compared with CAS-fed rats (Hakkak et al., 2000). We studied mammary gland morphology at age 50 days because it is important to understand the developmental status at the time when DMBA was administered. It is also important to determine which cellular systems are affected by these diets in order to ascertain the mechanisms underlying dietary protection against breast cancer. The decreased TEB density coupled with the increased expression of PR in rats fed SPI- or WPH-diets suggests these dietary factors are important stimulators of mammary gland differentiation. In PR gene knockout experiments there was no TEB to LOB differentiation (Lydon et al., 1999) implicating the PR as an important regulator for the conversion of the undifferentiated TEB into the more differentiated LOB. Increased numbers of cells expressing PR in the TEBs of rats fed SPI- or WPH-diets suggests that these structures may respond with increased sensitivity to the mammogenic effects of progesterone (Shyamala, 1999).

The differentiation status of the mammary gland has been proposed to affect tumor incidence since it is the undifferentiated highly proliferative TEBs that are the target of DMBA-induced tumors (Huggins and Yang, 1962; Russo and Russo, 1996); thus, lower TEB densities may result in lower tumor incidence. The reduced TEB density in the SPI- and WPH-fed rats suggests that this would be cancer protective. These effects are similar to those of rats fed the soy isoflavone, genistein (Lamartiniere et al., 1995). Female rats administered genistein during the prepubertal or neonatal stage had reduced TEB densities and treatment of these rats with DMBA at PND 50 resulted in fewer tumors and increased latency to tumor development (Lamartiniere et al., 1995; Murrill et al., 1996). In the present study, there was a four-fold decrease in mean TEB density in both SPI and WPH groups, but this did not reach statistical significance. Nonetheless, it suggests that this may be a contributing factor in lower incidence and multiplicity. Interestingly, the increased size of the mammary gland in the SPI-fed rats means that the absolute number of TEBs in these rats is not significantly different than in the CAS-fed rats; whereas the absolute number of TEBs in the WPH-fed rats is lower than in the CAS-fed rats, thus the reduced cancers in the SPI-fed rats appears to be the result of more than just SPI-induced increased gland differentiation.

In the SPI- or WPH-fed rats compared with CAS-fed rats there was reduced DMBA-induced expression of mammary CYP1A1, CYP1A2, and CYP1B1 mRNA and protein (Row-
lands et al., 2001). CYP1A1 and CYP1B1 are capable of activation of chemically diverse procarcinogens including DMBA (Shimada et al., 1996) and CYP1B1 of 4-hydroxylation of estradiol yielding a putative endogenous mutagen (Hayes et al., 1996; Liehr et al., 1995; Spink et al., 1994). Thus, the reduction of CYP1A1 and CYP1B1 expression in the SPI- and WPH-fed rats is consistent with reduced mammary cancer incidence measured in rats fed these diets. Moreover, the reduced CYP1A1 and CYP1B1 expression in the SPI-fed rats where there is functionally only a reduction of DMBA-induced CYP1A1 and CYP1B1 expression measured.

Increases in mammary gland size and PR expression are consistent with an estrogenic activity in the SPI-fed rats. Estrogen is a well-characterized inducer of PR expression (Mohla et al., 1981) and estrogens have been reported to induce the growth and area of rat mammary glands (Fendrick et al., 1998; Russo and Russo, 1996). Numerous estrogenic chemicals have been identified in soy (phytoestrogens) including genistein, daidzein, and sapogenol A (Adlercreutz and Mazur, 1997; Rowlands et al., in press). The increased PR expression in the WPH-fed rats is most likely not an estrogenic effect since WPH has not been shown to contain estrogens and there was no observed change in estrogen target tissues such as an increase in mammary gland size or uterine hypertrophy (Badger et al., 2001). Instead, the increased PR in the WPH-fed rats may have resulted from the actions of peptides generated in processing, or growth factors found in whey. For example,

<table>
<thead>
<tr>
<th>Diet</th>
<th>PCNA TEB</th>
<th>PCNA LOB</th>
<th>ERα TEB</th>
<th>ERα LOB</th>
<th>ERβ TEB</th>
<th>ERβ LOB</th>
<th>PR TEB</th>
<th>PR LOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td>29 ± 2.9</td>
<td>28 ± 6.4</td>
<td>41 ± 3.4</td>
<td>29 ± 2.7</td>
<td>59 ± 4.3</td>
<td>61 ± 2.2</td>
<td>30 ± 2.6</td>
<td>31 ± 2.7</td>
</tr>
<tr>
<td>SPI</td>
<td>23 ± 4.1</td>
<td>28 ± 4.8</td>
<td>40 ± 3.2</td>
<td>29 ± 5.8</td>
<td>61 ± 2.7</td>
<td>58 ± 3.4</td>
<td>40 ± 1.5*</td>
<td>36 ± 0.9</td>
</tr>
<tr>
<td>WPH</td>
<td>25 ± 2.9</td>
<td>28 ± 8.6</td>
<td>38 ± 4.2</td>
<td>29 ± 5.5</td>
<td>61 ± 1.2</td>
<td>56 ± 3.6</td>
<td>41 ± 3.5*</td>
<td>31 ± 3.7</td>
</tr>
</tbody>
</table>

*Note. Values are percent positively stained cells; means ± SEM.

*p < 0.05 CAS vs. SPI or WPH; (ANOVA; Student-Newman-Keuls multiple comparison).
whey contains IGF-1 (Grosvenor et al., 1993; Guimont et al., 1997) and PR was shown to be induced by IGF-I in vitro (Cho et al., 1994).

In summary, the results reported here have revealed that in rats fed SPI- or WPH-based diets there is increased PR expression and mammary gland differentiation compared with rats fed a CAS-diets. The mechanisms for the SPI- and WPH-induced changes are not known, but for SPI they may in part be due to the phytoestrogens since there were also increases in mammary gland size and estrogens can cause this effect. The WPH-induced mammary gland differentiation may be due to the mammogenic peptides such as IGF-1 in whey or may be due to peptides generated from processing or digestion. The increased differentiation is a potential mechanism for the decreased mammary cancer measured in SPI- or WPH-fed rats compared with CAS-fed rats.

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Diet and Mammary Gland Development


