Proliferative Activity of Murine Mammary Epithelium as Affected by Dietary Fat and Calcium

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ABSTRACT

Dietary fat and calcium have been found to affect significantly the proliferative status of the mammary glands. Female mice (3-week-old C57BL/6J) were given either a low or high corn oil diet (3 or 30% by weight). One, 2, or 4 weeks after the dietary intervention the animals were given injections of \(^{3}H\)thymidine and/or colchicine; 2 h later their thoracic mammary glands were removed and processed for histology and autoradiography. Animals on the high corn oil diet had an increased labeling index of both terminal ducts and mature ducts compared to the control group at each time (i.e., 10.1 ± 2.1 versus 4.8 ± 0.9% at 2 weeks). This effect of a high corn oil diet was evident on the mammary glands of animals at various ages. Animals on a high beef tallow diet also had a high labeling index. This effect of a high fat diet appeared to be reduced quantitatively increases the proliferation of mammary epithelial cells. The tritiated thymidine labeling of mammary epithelium increased in mice receiving diets containing 0, 5, or 20% CO. The study was carried out with 7 groups of mice aged 3 weeks, for periods of from 0 to 4 weeks on the diets. In Study 2, groups of mice of 8 and 13 weeks of age were placed on either low or high fat diets (30% CO or 30% CO plus 27% BT) and the proliferative status was assessed 4 weeks later when the animals were 12 and 17 weeks of age. In Study 3, low and high fat diets containing graded levels of calcium (0.1, 0.5, or 1.0%) were compared as previously described. The mice were fed these diets from an age of 3 weeks for 4 weeks.

INTRODUCTION

Welsch et al. (1) recently observed that dietary fat as corn oil quantitatively increases the proliferation of mammary epithelial cells. The tritiated thymidine labeling of mammary epithelium increased in mice receiving diets containing 0, 5, or 20% CO for the period from the third week to the third month of their lives. This observation is interesting in light of the well known influence of dietary fat on the development of the mammary gland and on its sensitivity to breast carcinogens (2-7), and of the evidence linking diet and human breast cancer (8, 9). It thus seemed important to determine whether the proliferative effect of fat could be seen over shorter periods of time, over later periods in the lives of the animals, with fats other than CO, or in association with other dietary variables.

MATERIALS AND METHODS

Animals. Female C57BL/6J mice were supplied by The Jackson Laboratory (Bar Harbor, ME) at 3 weeks or 7 weeks of age. Prior to the diet interventions the animals were fed mouse chow ad libitum. During this period and throughout the study they had access to chlorinated water (3-5 ppm). The animals were housed in wire-top cages with sawdust bedding, with a 12-h light, 12-h dark cycle. After a short acclimatization period the stock animals were randomized into groups of about 9 animals as detailed in Table 1. This table details the ages of the animals used in each of the three sections of "Results." These studies demonstrate that a high fat diet affects the proliferative status in the mouse mammary glands in a short period of time and that this effect can be reduced by dietary calcium.

Diet. The formulated diets were based on the AIN-76 diet (10) with modifications for type of carbohydrate and the level of fat and calcium as shown in Table 2. The diet was available to the animals at all times. The low fat diet contained 3% CO (Mazola corn oil) by weight. In order to make a high fat diet (30% by weight CO or BT), additional fat (CO or BT) was added to the low fat diet by replacing the isocaloric amount of dextrose. BT was purchased from Hubbert's Processing (Mississauga, Ontario, Canada). Calcium levels were altered by modifying the levels of calcium phosphate (CaHPO\(_4\)) in the mineral mix as described previously (11, 12) to hold the calcium:phosphate ratio at 1:0.

Study 1 compared the effect of diets containing either 3 or 30% CO. The study was carried out with 7 groups of mice aged 3 weeks, for periods of from 0 to 4 weeks on the diets. In Study 2, groups of mice of 8 and 13 weeks of age were placed on either low or high fat diets (30% CO or 30% CO plus 27% BT) and the proliferative status was assessed 4 weeks later when the animals were 12 and 17 weeks of age. In Study 3, low and high fat diets containing graded levels of calcium (0.1, 0.5, or 1.0%) were prepared as described previously. The mice were fed these diets from an age of 3 weeks for 4 weeks.

Measurement of Proliferative Indices. The animals were given injections of colchicine (1 \(\mu\)g/g body weight) and \(^{3}H\)thymidine (2 \(\mu\)Ci/g body weight, 42 Ci/mmol; Amersham Corp., Arlington Heights, IL) 2 h before they were killed by cervical dislocation. The two anterior mammary glands were then stretched out flat on a firm filter paper, fixed, embedded, and whole-mount cut so that sections contained representative samples of the entire gland from the ducts below the nipple to the lateral terminal structures. Three step sections were prepared for autoradiography by traditional methods (11, 12) and after development were stained with hematoxylin and eosin.

These slides were coded and examined, first under low power to select at random well defined terminal and mature structures (Fig. 1, A), and then under oil immersion to count the number of labeled, mitotic, and total cells in each structure (Fig. 1, B and C). Five to 8 terminal and ductal structures were examined with each step section of both of the breasts for the 8 to 10 animals per group. Results were given as labeling index or mitotic index.

RESULTS

Duration of Low and High Fat Feeding on the Proliferative Indices of Mammary Glands (Study 1). As noted above, the studies of Welsch et al. (1) were made in animals on different diets for the period from 3 weeks to 3 months. Our first study examined the effect of the duration of dietary differences of 3 and 30% CO. The study was carried out with 7 groups of mice aged 3 weeks, for periods of from 0 to 4 weeks on the diets.

The initial labeling index of the cells in the terminal structures was 3.6% at 3 weeks (Fig. 2). After 1 week this rose to 6.5% for the animals on the low and to 9.4% for animals on the high fat diets. The difference appeared even more evident after 2 and 4 weeks when the difference was approximately 2-fold. Similar differences were seen with mitotic figure measures of proliferation (data not shown). The first study thus confirmed that a high fat diet leads to an increased proliferation rate in the mammary epithelium. As the increase was clearly evident in the terminal structures at 4 weeks, subsequent studies were made at this interval.

Effect of a Low and a High Fat Diet on the Proliferative Indices in Mice of Various Ages (Study 2). The mammary tree of the mouse differs greatly over its life. To determine whether the effect of fat on proliferation was limited to only the early period or extended over a longer time, we compared the effects of low and high fat diets over periods into the maturity of the animals.
The results (Fig. 2) make it clear that the dietary effects of fat are evident over a prolonged period in the lives of the animals. However, the effect was most pronounced in the terminal structures of the mammary glands and therefore was most evident in the younger animals.

Effect of High BT and CO Diets Containing Calcium (Study 3). The effects of these dietary variables on the proliferation of the young animals was next examined. Low and high fat diets containing graded levels of calcium (0.1, 0.5, or 1.0%) were prepared with dietary fat levels of 3% CO, 30% CO, or 3% CO together with 27% BT as described previously. The mice were fed these diets from the age of 3 weeks for a period of 4 weeks.

The body weights of the animals on high fat diets were higher ($P < 0.05$) than those on the low fat diet (Fig. 3). Animals on the lowest level of calcium with a low fat diet appear to be higher in body weight than their higher calcium counterparts (Fig. 4A), whereas the animals on high fat diets had similar body weights irrespective of the level of calcium (Fig. 4, B and C). However, none of these differences was statistically significant.

It is evident from the results (Fig. 5) that BT produces a similar increase in proliferation to that seen with CO. The proliferative indices measured as labeling index (Fig. 5A) or mitotic index (Fig. 5B) seen with 27% BT and 3% CO were higher than that seen with 3% CO, and the rate was approximately the same as that seen with 30% CO. An effect of calcium was also evident. Animals receiving diets with 0.1% calcium

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**Table 1 Summary of experimental protocols**

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<tr>
<th>Study</th>
<th>Group</th>
<th>Dietary fat (%)</th>
<th>Calcium (%)</th>
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<th>Diet duration (wk)</th>
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<td>3</td>
<td>3 CO</td>
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<tr>
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<td>7</td>
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**Table 2 Composition of experimental diets**

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<th>% of composition*</th>
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<th>High fat</th>
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<td>Casein</td>
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<td>Dextrose</td>
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<td>Cellulose</td>
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<tr>
<td>Vitamin mix</td>
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<td>1.3</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Low fat, 3% CO; high fat, 30% CO or 3% CO plus 27% BT.
*AIN-76 mineral mix was modified for 0.1 and 1% calcium diets as described previously.
*AIN-76 vitamin mix.
DIETARY FAT AND PROLIFERATION OF MAMMARY EPITHELIUM

Fig. 2. Effect of low (3%) and high (30%) corn oil diet on the proliferative indices of terminal ducts of mouse mammary epithelium at various ages.

Fig. 3. Body weights of animals on various diets for up to 4 weeks. 3% corn oil (△), 30% corn oil (●), or 3% corn oil plus 27% beef tallow (■).

Fig. 4. Body weights of animals on diets varying in the level and type of fat and the level of calcium. A, 3% CO; B, 30% CO; C, 3% CO plus 27% BT. Curves A, D, and G, 0.1% calcium; Curves B, E, and H, 0.5% calcium; Curves C, F, and I, 1.0% calcium.

had significantly higher levels of proliferation than animals in the 0.5 and 1.0% groups. This effect was much more pronounced in the terminal structures of the mammary gland of high fat groups (Fig. 5, top) than in the mature ducts (Fig. 5 bottom).

DISCUSSION

The results of these studies confirm and extend the observations made by Welsch et al. (1). Under all the conditions of our studies, the proliferation of the mammary epithelium of animals on high fat diets (30% CO by weight) was higher than that seen in animals on low fat diets (3% CO). It was seen in animals over periods from 1 to 4 weeks for animals of 3 to 17 weeks of age. Proliferation was observed in both the ductal cells as well as the terminal structures and was evident for both the high CO diet and BT diet. The results also showed that dietary calcium reduced the proliferation of the mammary epithelium, especially in animals on high fat diets.

In the present study we have not made any attempt to classify the development of mammary glands for the various groups. Increased proliferative indices indicating enhanced growth were seen with the higher levels of fat in both the terminal as well as central ductal structures at each of the times at which animals were examined from 4 to 17 weeks of age. The elevated levels in terminal structures are probably most germane to sensitivity to carcinogenesis, as these structures appear to be the most sensitive to the exposure to carcinogens and the development of adenocarcinoma (5, 13, 14).

The effect of the BT and CO diets on the proliferative response is of interest, given the attention paid to the effect of unsaturated fatty acids on mammary tumorigenesis and on the growth and development of mammary glands. Our observations suggest that animal fat when supplemented with sufficient linoleic acid provides the proliferative stimulus equivalent to vegetable fat. This result is consistent with carcinogenesis studies that have observed a promoting effect of diets containing n-6 polyunsaturated fats (15, 16). Diets high in n-3 fatty acids may behave differently (17, 18).

Dietary calcium as CaHPO₄ both in diets low and high in dietary fat appeared to reduce the proliferative indices of mammary glands. The result is intriguing, given that dietary calcium produces a similar reduction in the proliferative status of colonic epithelium (11, 12, 19, 20). The reduction in the case of the colon has generally been attributed to effects of calcium on the solubility of fatty acids and bile acids in the colonic lumen (21, 22), but such a model does not help to explain the effects in the breast. Perhaps the simplest explanation is that high levels of dietary calcium reduce the absorption of fat (23). It is
also possible that calcium has its effects through regulatory pathways in proliferation (24).

The observed effect of dietary fat on the proliferation of cells in the breast is very striking. The phenomenon is sufficiently large and rapidly apparent as to make it possible to consider the design of experiments aimed at an understanding of the mechanism leading from diet to cell proliferation, and perhaps also from cell proliferation to cancer promotion and carcinoma.

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REFERENCES

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