EFFECTS OF DIETARY FAT ON THE GROWTH OF NORMAL, PRENEOPLASTIC AND NEOPLASTIC MAMMARY EPITHELIAL CELLS IN VIVO AND IN VITRO

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SUMMARY

In order to determine: (1) whether there is a growth-regulating interaction between the mammary fat pad and mammary epithelium; (2) whether this interaction could be modified by dietary fats; and (3) whether these effects could be demonstrated in vitro, the following experiments were performed. Virgin Balb/c mice had the left inguinal mammary fat pad cleared of epithelium and were then maintained on one of four fully defined diets. These diets contained the following proportions of fat by weight: 5% or 10% mixed fats; 20% saturated fat plus cholesterol; or 20% polyunsaturated fat. To test for effects in vivo, animals received subcutaneous injections into the cleared fat pad of tumorigenic mammary cells (WAZ-2T(+SA) or WAZ-2T(-SA)) or preneoplastic mammary cells (CL-S1). Dietary fat had little effect on the latent period of tumour formation, but a low-fat diet increased the invasive/metastatic potential of both tumorigenic cell lines. A high-saturated-fat diet inhibited the growth of normal and preneoplastic epithelium in vivo. To test for effects in vitro, CL-S1 cells were co-cultured with explants of cleared mammary fat pad embedded within collagen gels. CL-S1 cells co-cultured with adipose explants obtained from mice fed on a diet containing 20% polyunsaturated fat showed a threefold increase in incorporation of [3H]thymidine into trichloroacetic acid-precipitable material. These results imply that dietary fats may affect the growth of mammary epithelium in two ways: the inhibition of growth caused by the high-saturated-fat diet may be due to systemic effects as it was not apparent in vitro; the increase in growth seen in vitro and caused by a high-polyunsaturated-fat diet is due to a direct interaction between the mammary fat pad and mammary epithelial cells. This interaction may be masked by systemic effects in vivo.

INTRODUCTION

Many studies have shown a correlation between dietary fats and mammary tumour incidence in both humans (see review by Bandaru et al. 1980) and experimental animals (Hillyard & Abraham, 1979; Gridley, Kettering, Slater & Nutter, 1983). Although responses to specific components vary, in general a diet that contains a high proportion of fat has been shown to increase tumour incidence or the rate of tumour growth, whereas a fat-free or low-fat diet may have a protective effect (Chan, Head, Cohen & Wynder, 1977).

A classic observation in mammary cell biology was the discovery that normal mammary epithelium will proliferate only if it is embedded in a matrix of white

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adipose tissue (Hoshino & Martin, 1974). Since then, the adipose–epithelium interaction has been considered by several groups as it applies to both normal and neoplastic growth (Beer & Billingham, 1978; Miller, Medina & Heppner, 1981; Bartley, Emerman & Bissell, 1981). We wished to determine whether there is a direct, local growth-regulating interaction between the fat pad and normal preneoplastic and neoplastic epithelial cells that could be modified by dietary fats. We have also analysed whether such modifications of the local interaction can be demonstrated in vitro and thus clearly distinguished from systemic effects on mammary epithelial proliferation.

MATERIALS AND METHODS

Animals and diets

Virgin female Balb/c mice, aged 3 to 3½ weeks were obtained from D. Medina and from our colony maintained in the Eastlick Vivarium, Washington State University. All the mice used in this study had the left inguinal fatpad cleared of epithelium by the method of DeOme, Faulkin, Bern & Blair (1959) before the age of 4 weeks. Fully defined diets were composed as shown in Table 1. For details of fatty acid compositions of the oils used in this study see Tinsley, Schmitz & Pierce (1981). The prepared diets were stored at −20°C until fed to the mice. Completely fresh food, −25 g per cage of four animals, was given to the mice daily.

Culture techniques

WAZ-2T(+)SA, WAZ-2T(-SA) and CL-S1 cells (Danielson, Anderson & Hosick, 1980; Anderson, Danielson & Hosick, 1979) were grown as monolayers and trypsinized briefly to remove them from the culture dishes. They were then washed and either resuspended in sterile isotonic saline and injected into mice, or they were resuspended in collagen gel solution, which was

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control</th>
<th>Low fat</th>
<th>High saturated fat plus cholesterol</th>
<th>High poly-unsaturated fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>49</td>
<td>54</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Casein*</td>
<td>25</td>
<td>25</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Cellulose*</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt mix†</td>
<td>4</td>
<td>4</td>
<td>3-5</td>
<td>3-5</td>
</tr>
<tr>
<td>Vitamin mix†</td>
<td>2</td>
<td>2</td>
<td>1-8</td>
<td>1-8</td>
</tr>
<tr>
<td>L-methionine*</td>
<td>0-1</td>
<td>0-1</td>
<td>0-1</td>
<td>0-1</td>
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<tr>
<td>Safflower oil*</td>
<td>2-5</td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Coconut oil†</td>
<td>2-5</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Cottonseed oil†</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol*</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Bile salts</td>
<td></td>
<td></td>
<td>0-4</td>
<td></td>
</tr>
<tr>
<td>Total fat</td>
<td>10</td>
<td>5</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

* Obtained from Sigma Chemical Company.
† Purchased from ICN.
immediately plated. Collagen gels were prepared as described by Yang et al. (1980). Explants (1 mm³) of cleared mammary fat pad were prepared by sterile microdissection of fresh tissue. They were plated, 3 per 30 mm culture dish, and immediately overlaid with 2-5 ml of collagen gel solution, or 2-5 ml of collagen gel solution containing suspended Cl-S1 cells. After they had set, the gels were overlaid with 1 ml of Dulbecco’s MEM supplemented with newborn bovine serum (5 %, v/v) and insulin (5 μg/ml). Oestrogen (2 ng/ml) was added as an ethanolic solution. An equal volume of ethanol was added to the control medium and did not exceed 0.1 % (v/v). The cultures were incubated in a 95 % O₂/5 % CO₂ atmosphere and the culture medium was replaced every 48-72 h.

**Measurement of cell growth**

The incorporation of [³H]thymidine into trichloroacetic acid (TCA)-precipitable material was used to measure the growth of cells in collagen gel culture. Rate of incorporation has been shown to correlate well with subsequent increases in cell number and total DNA (unpublished data). The culture medium was replaced with 1 ml of fresh medium containing 2 μCi of [³H]thymidine and incubation at 37 °C was continued for 24 h. The gel was then rinsed three times with ice-cold saline, cooled rapidly and maintained at 4 °C for at least an hour. The final rinse was then removed and the gel melted at 50 °C on a slide-warmer. The molten gel plus suspended cells were transferred to a centrifuge tube and the tissue pelleted by centrifugation at 1000 rev./min for 10 min. All but 0.5 ml of the supernatant was removed by aspiration. The tissue was disrupted in a bath sonicator and then cooled at 4 °C. The DNA and protein were precipitated with cold trichloroacetic acid (5 %, v/v) and separated from any remaining TCA-soluble radioactivity by filtration onto glass fibre filters (Whatman, GF/C). The filters were dried, immersed in 3a7ob scintillation fluid and the radioactivity was determined on a Packard Tri-Carb Scintillation counter.

**Experimental protocol**

Beginning 10 days after the operation to clear the mammary fat pad, groups of mice were maintained on each of the four fully defined diets. After a further 30 days each group of mice was subdivided into: three groups of four mice each for *in vivo* experiments; and one pair that was used as a source of tissue for *in vitro* experiments. All the mice were weighed weekly and checked for signs of essential fatty acid deficiency.

**Experiments in vivo.** After 30 days on the fully defined diets, groups of four mice received injections (0.1 ml) of 1×10⁶ cells of the CL-S1, WAZ-2T(+SA), or WAZ-2T(−SA) cell lines into the cleared mammary fat pad (i.e. 12 treatments). The remaining mice (two per diet) served as non-injected controls and it was these that were later used as a source of tissue for the culture experiments. All animals were palpated weekly and the times of formation of palpable tumour were recorded. Injected mice were maintained for 4 months or until death resulted. Mammary tumours were excised and weighed and any other tumours were noted post mortem. Four months after injection, the surviving injected mice were killed and the left and right inguinal mammary glands were whole-mounted and stained with Mayer’s haematoxylin.

**Experiments in vitro.** CL-S1 cells (5×10⁴ per dish) and explants (three per dish) of cleared mammary fat pad obtained from mice on fully defined diets were cultured within collagen gels separately, and as co-cultures, in the presence and absence of oestrogen (2 ng/ml). Growth was assessed on the 10th day of culture. In order to compare the incorporation of [³H]thymidine by CL-S1 cells cultured alone with that of CL-S1 cells co-cultured with mammary fat pad explants, the following calculations were made for each experiment.

In each of *i* cultures labelled, let incorporation of [³H]thymidine by: CL-S1 cells = a; adipose explants = b; co-cultures = c.

For each experiment let: \( \frac{\sum a}{n} + \frac{\sum b}{n} = 1.00 \).

Then compare \( \frac{c_i}{(a + b)} \) with \( \frac{(a_i + b_i)^*}{(a + b)} \).

* Results paired randomly.
We have assumed in this work that the increase in incorporation results primarily from increased DNA replication by the CL-S1 cells, since mature adipocytes do not replicate. The non-adipose cells of explants likewise do not replicate in the intact adult adipose tissue; the 'fibroblastoid' cells that migrate out of explants replicate poorly in collagen gels (Buttle & Ehrlich, 1983) and we have not observed mitoses in this cell type.

RESULTS

Fully defined diets

All four diets supported a good rate of growth in recipient mice (Fig. 1) and none of the animals showed any symptoms of essential fatty acid deficiency. All the mice were clean and healthy in appearance except those on the high-saturated-fat plus cholesterol diet, which developed large deposits of abdominal fat and did not groom.

Fig. 1. Growth of mice on fully defined diets. Each point represents the average of all mice on the indicated diet; variation between mice was less than 5 %. (○—○) Control diet; (●—●) low-fat diet; (□—□) high-saturated-fat diet; (■—■) high-unsaturated-fat diet.
Fat and growth of mammary epithelium

Experiments in vivo

All the mice injected with the WAZ-2T(+SA) cell line developed mammary tumours. The average tumour weight at the time of death was 7.2 ± 2.9 g. There was no significant effect of dietary fat on tumour latency (Table 2). In all but one mouse the mammary tumour had invaded the peritoneum and tumours were also found at other sites in the abdominal cavity (in 8/15 mice), particularly along the intestines. Table 3 shows how invasion/metastasis was affected by dietary fat.

All the mice injected with the WAZ-2T(-SA) cell line also developed mammary tumours (average weight = 6.0 ± 2.5 g). Table 2 shows that again there was no significant effect of dietary fat on the length of the latent period. The (-SA) cell line is less invasive than the (+SA) line (Danielson et al. 1980) and hence mammary tumours that had invaded the peritoneum were found in only 5/15 mice, and tumours at other sites in 4/15 mice. Table 3 shows that in this line also there was a correlation between low dietary fat and increased invasion.

None of the animals injected with the preneoplastic, CL-S1 cell line developed tumours within 4 months. They were, therefore, killed, both left and right inguinal fat pads were mounted and stained, and each pair of glands was graded according to the

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>Latent period of tumour formation (days)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WAZ-2T(+SA)</td>
</tr>
<tr>
<td>Control</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Low</td>
<td>33 ± 18</td>
</tr>
<tr>
<td>High saturated</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>High unsaturated</td>
<td>23 ± 8</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.E.M. of four animals except, * mean ± S.E.M. of three animals.

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>No. of animals with tumours in sites other than mammary fat pad</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WAZ-2T(+SA)*</td>
</tr>
<tr>
<td>Control</td>
<td>1/4</td>
</tr>
<tr>
<td>Low</td>
<td>4/4</td>
</tr>
<tr>
<td>High saturated</td>
<td>1/3Ⅰ</td>
</tr>
<tr>
<td>High unsaturated</td>
<td>2/4</td>
</tr>
</tbody>
</table>

• Tumours in sites other than mammary fat pad and peritoneal wall.
† Tumours in sites other than mammary fat pad.
§ One mouse badly chewed, dissection impossible.
Ⅰ One mouse killed accidentally before tumour developed.
extent of epithelial development (Fig. 2). There was little or no difference between the control gland, and the cleared and injected fat pad in the same animal. It is clear from Fig. 2 that the diet that contained a high proportion of saturated fat inhibited the growth of the injected non-tumorigenic cells and the endogenous normal mammary epithelial cells.

Experiments in vitro

There was no significant effect of oestrogen on the incorporation of \[^3\text{H}\]\text{thymidine} by CL-S1 cells or by explants of mammary fat pad under any of the conditions tested. However, there was a non-significant decrease in incorporation of \[^3\text{H}\]\text{thymidine} by adipose explants cultured in the presence of oestrogen, both alone and in co-culture with CL-S1 cells.

It can be seen from Table 4 that a significant effect of co-culture on the incorporation of \[^3\text{H}\]\text{thymidine} occurs when the explants are derived from mice maintained on a diet that contains a high proportion of unsaturated fatty acids \((P<0.05\text{ by Student's } t\text{-test})\). With other diets, the data suggest an interaction between adipose explants and CL-S1 cells, but the interaction has considerably less effect on growth.
Conclusions

The fully defined diets used in this study were chosen to reflect 'normal' rather than 'experimental' dietary regimes. Particular care was taken to ensure that all the animals, especially those on the low-fat diet, were given adequate amounts of the essential fatty acids.

The proportion and type of dietary fat had no significant effect on the latency of mammary tumour formation by WAZ-2T(+SA) or WAZ-2T(-SA) cells injected into Balb/c mice. Neither was any effect observed on the growth rate of the tumours as estimated by the increase in tumour diameter (data not shown). This agrees with the report by Hillyard & Abraham (1979) that linoleate (C_{18:2}) is equally effective in promoting mammary tumour growth in Balb/c mice at concentrations ranging from 0·1 %–15 % of the diet, as in the present study the levels of linoleate were 0·9 %–16 % of the diet.

However, the low-fat diet (5 %, w/w) increased the invasive/metastatic potential of both cell lines. Because the high-polyunsaturated-fat diet did not have this effect it is unlikely to have been caused by the immunosuppressive effect of polyunsaturated fatty acids (Mertin & Hunt, 1976; Kollmorgen, King, Kosanke & Do, 1983). It is possible that a low fat diet may alter the lipid composition of the cell membranes of the tumour and/or the host tissues, such that their fluidity and hence the invasive potential of the tumour is increased. Since the object of this study was the comparison of the interactions between mammary fat pad and mammary epithelium in vivo and in vitro, these results were not investigated further during this project.

The responses to dietary lipid that we describe for mice are different in several ways from those noted for rat and human. Previous authors have pointed out that responses to polyunsaturated fatty acids depend on species of animal (Hillyard, Rao & Abraham, 1980), and even on the strain of mouse (Hillyard & Abraham, 1979) or rat (Chan et al. 1977). Discrepancies may in part be explained by subtle differences between diets; for example, in the concentrations of cyclopropenoid fatty acids (Tinsley, Wilson & Lowry, 1983) and differences in sources of dietary proteins (Gridley et al. 1983). However, there may be genuine species differences in response

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>Control medium</th>
<th>Medium plus oestrone (2 ng/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Separate</td>
<td>Co-culture</td>
</tr>
<tr>
<td>Control</td>
<td>1·00 ± 0·37</td>
<td>1·34 ± 0·90</td>
</tr>
<tr>
<td>Low</td>
<td>1·00 ± 0·17</td>
<td>1·36 ± 0·41</td>
</tr>
<tr>
<td>High saturated</td>
<td>1·00 ± 0·23</td>
<td>1·83 ± 1·22</td>
</tr>
<tr>
<td>High unsaturated</td>
<td>1·00 ± 0·40</td>
<td>3·04 ± 1·67</td>
</tr>
</tbody>
</table>

Mean ± s.e.m. (n = 6); two independent experiments each performed in triplicate.
to free radicals generated by polyunsaturated fatty acids, for example, or in the
significance of prostaglandins (for which linoleate is a precursor via arachidonate) in
tumour promotion (Rao & Abraham, 1976; Abraham, Faulkin, Hillyard & Mitchell,
1984). Both saturated and unsaturated fatty acids may affect levels of circulating
prolactin; rat and human mammary tumours seem particularly susceptible to levels of
lactogenic hormone (Bandaru et al. 1981; Cave & Erickson-Lucas, 1982) in
comparison to mouse tumours (Hosick & Nandi, 1974). Recent evidence, however,
casts some doubt on the ability of dietary fats to alter circulating levels of mammogenic
hormones in rats (Welsch & Aylsworth, 1983; Wetsel, Rogers, Rutledge & Leavitt,
1984). Nonetheless, a reasonable hypothesis would be that the mechanisms by which
dietary lipids hasten mammary tumorigenesis probably are dissimilar in several
groups of mammals.

There are several possible explanations for the lack of difference in epithelial
growth between the intact mammary glands and those cleared of epithelium and
subsequently injected with CL-S1 cells: (1) the fat pad was cleared of epithelium and
the injected CL-S1 cells grew in a similar fashion to normal mammary epithelium;
(2) the fat pad was not cleared properly so native epithelium proliferated and the
injected cells failed to grow. Although no cleared glands that had not been injected
with CL-S1 cells were fixed and stained, the explants of cleared mammary fat pad
used in the co-culture system showed no evidence of epithelial outgrowth. Thus the
first explanation is the more likely. It is interesting to note that growth of the intact
parent hyperplastic outgrowth line (D1) from which the CL-S1 cell line was isolated is
relatively insensitive to dietary lipid in vivo (Abraham et al. 1984). Taken together
with our observation, that a cell population isolated from these nodules (CL-S1) is
responsive to the adipose environment, this lends credence to the concept that multi-
cellular architecture is an important component of growth properties of preneoplastic
nodules (Medina, Shepherd & Gropp, 1978).

The slight inhibition of normal epithelial development seen in the mice fed the
high-saturated-fat diet may help explain the increased frequency of tumour
development observed in such animals (Tinsley et al. 1981) since normal mammary
epithelial cells have been reported to inhibit the growth of neoplastic mammary cells
(Faulkin & DeOme, 1960; Medina, Shepherd & Gropp, 1978). The mechanism by
which this diet inhibits the growth of normal epithelium is not yet known. However, it
seems probable that the effect is either systemic or direct, and not mediated by the
mammary fat pad, since the inhibition was not observed in co-cultures in vitro. In
contrast, a stimulation of growth was seen when CL-S1 cells were co-cultured with
mammary fat pad explants from mice fed the high-polysaturated-fat diet. This
stimulation must have been due to an interaction between the explants and the
epithelium, thus showing that such interactions occur and can be modified by the type
and quantity of fat in the diet. In addition to stimulation of epithelium by adipose
tissue, the converse has also been shown, i.e. local stromal DNA synthesis can be
stimulated by nearby epithelium (Berger & Daniel, 1983). Because the epithelial cells
(CL-S1) were identical in all the in vitro studies reported here, differences in adipose
response during culture seem unlikely. Any intrinsic differences in rates of stromal
DNA synthesis on different dietary regimens are taken into account by our method of calculation of results.

The culture system described can be used to differentiate between systemic and intraglandular effects of diet and other adipose-mediated responses of epithelium and should greatly facilitate such studies.

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REFERENCES


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