A Role of Estrogens and Insulin Binding in the Dietary Lipid Alteration of R3230AC Mammary Carcinoma Growth in Rats

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ABSTRACT

The importance of estrogens in the dietary lipid alteration of R3230AC mammary carcinoma growth and insulin binding was studied. Animals were divided into three groups [intact, ovariectomized, and ovariectomized treated with estradiol valerate (EV)] and were fed diets containing either 0% fat (fat free), 0.5% corn oil (low fat), or 20% corn oil (high fat). An alteration of tumor burden between animals fed high-fat versus either low-fat or fat-free diets was observed and appeared to be influenced by the estrogen status of the animal. The difference in tumor burden attributed to dietary lipid seen in intact rats was less in ovariectomized rats and greater in ovariectomized rats treated with EV, despite the fact that absolute tumor burden was reduced by this treatment. A similar relationship was observed for dietary lipid-induced differences in insulin binding to plasma membranes from these tumors. Reduction of tumor growth resulting from estrogen treatment was greater in low-fat- and fat-free-fed animals than in high-fat-fed rats. Again, tumor growth behavior appeared to be related to the reduction in insulin binding induced by estrogen treatment; insulin binding to plasma membranes from animals fed a low unsaturated lipid diet was decreased to a greater extent by EV treatment than in membranes from high-fat-fed rats. Altered tumor growth and membrane insulin binding, resulting from dietary perturbations and/or EV treatment, were not invariably related to serum insulin levels, nor to differences in membrane preparation, as reflected by 5'-nucleotidase activity, nor to membrane fatty acid composition or uptake of proline. Taken together, these results suggest a potential role of estrogens and insulin receptors as mediators of the dietary lipid alterations of growth of the R3230AC mammary carcinoma.

INTRODUCTION

Numerous reports have appeared implicating dietary lipids as one factor responsible for enhanced mammary tumor development in both chemically and transplanted tumor models (11, 22, 29, 34, 46). Although the mechanism(s) responsible for these observations remain unknown, several investigators have sought out perturbations in the hormonal milieu as possible mediators of the dietary effects, i.e., changes in prolactin and estrogen levels. Chan and coworkers (13, 14) attributed enhanced growth of DMBA*-induced mammary tumors, resulting from the feeding of a HF diet, to an increase in serum prolactin levels. However, Aylsworth et al. (4) and Wetsel et al. (55) were unable to demonstrate an elevation in serum prolactin levels in HF-fed rats at any stage of the estrous cycle. Furthermore, since blood samples were obtained via indwelling cannulae, prolactin release due to stress was prevented (7). However, Caeve and Erickson-Lucas (12) reported that the prolactin binding capacity of microsomes from N-nitroso-N-methylurea-induced mammary tumors in HF-fed animals was significantly elevated compared to that seen in tumors from LF-fed rats, implying a possible role for prolactin. Neither Wetsel et al. (55) nor Ip and Ip (35) observed alterations in serum estradiol in animals ingesting diets high in fat content. Furthermore, Aylsworth et al. (3) demonstrated that, in ovariectomized rats maintained on uniform circulating levels of estradiol and prolactin, dietary lipids altered growth of DMBA-induced tumors. Thus, it appears that the enhancement of growth of these carcinogen-induced mammary tumors produced by elevated dietary lipid may not simply be due to elevated circulating levels of prolactin or estradiol, hormones known to be essential for growth of these experimental models. Hormone binding capacity at the cellular level, however, may play a role.

Few investigations have been directed towards an examination of the relationship between dietary lipid intake and the responses of mammary tumors to an altered estrogenic milieu. Difficulties are encountered when undertaking such studies with the above carcinogen-induced mammary tumor models, which are ovarian dependent for tumor induction as well as for progression of growth. In one study (16), it was reported that HF diets enhanced tumorigenesis of DMBA-induced tumors in ovariectomized rats, in which ovariectomy and dietary alterations were initiated at 80 and 87 days, respectively, after administration of the carcinogen. A more recent study (3) concluded that HF diets did not enhance mammary tumorigenesis of DMBA-induced tumors in ovariectomized rats. In order to explore the possible influence of dietary lipids on estrogen-induced alterations in growth and biochemical properties of mammary tumors, we undertook a study with the R3230AC transplatable mammary carcinoma. This model system appeared to be well suited, since Hillyard and Abraham (29) had reported that growth of the R3230AC tumor was more rapid in rats fed 15% corn oil-containing diets compared to those fed FF diets. Further, this well-characterized, hormone-responsive neoplasm grows as well in ovariectomized and intact hosts (27) and thus was amenable for a study of estrogen-induced responses.

Based on the observation that dietary lipids produce alterations in plasma membrane fatty acid composition, it is possible that such alterations could affect the binding of insulin (23, 24), a hormone that has been implicated as a factor regulating growth of this tumor (25, 26). Further, alterations in membrane composition could lead to altered transport of various substrates (10, 19, 24, 31, 39) and signal transduction (2, 3, 8, 20, 40, 44). The importance of estrogens in the dietary lipid alteration of R3230AC mammary carcinoma growth and insulin binding was studied. Animals were divided into three groups [intact, ovariectomized, and ovariectomized treated with estradiol valerate (EV)] and were fed diets containing either 0% fat (fat free), 0.5% corn oil (low fat), or 20% corn oil (high fat). An alteration of tumor burden between animals fed high-fat versus either low-fat or fat-free diets was observed and appeared to be influenced by the estrogen status of the animal. The difference in tumor burden attributed to dietary lipid seen in intact rats was less in ovariectomized rats and greater in ovariectomized rats treated with EV, despite the fact that absolute tumor burden was reduced by this treatment. A similar relationship was observed for dietary lipid-induced differences in insulin binding to plasma membranes from these tumors. Reduction of tumor growth resulting from estrogen treatment was greater in low-fat- and fat-free-fed animals than in high-fat-fed rats. Again, tumor growth behavior appeared to be related to the reduction in insulin binding induced by estrogen treatment; insulin binding to plasma membranes from animals fed a low unsaturated lipid diet was decreased to a greater extent by EV treatment than in membranes from high-fat-fed rats. Altered tumor growth and membrane insulin binding, resulting from dietary perturbations and/or EV treatment, were not invariably related to serum insulin levels, nor to differences in membrane preparation, as reflected by 5'-nucleotidase activity, nor to membrane fatty acid composition or uptake of proline. Taken together, these results suggest a potential role of estrogens and insulin receptors as mediators of the dietary lipid alterations of growth of the R3230AC mammary carcinoma.

1 Supported by USPHS Grant CA16660, NIH. Presented in part at the 75th Annual Meeting of the American Association for Cancer Research (19).
2 Portions of this material come from the dissertation to be submitted in partial fulfillment of the Ph.D. degree.
3 To whom requests for reprints should be addressed, at Department of Biochemistry, Box 607, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.
4 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; EV, estradiol valerate; FF, fat-free; LF, low-fat; HF, high-fat; HCTO, hydrogenated cottonseed oil; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
5 Received 7/9/84, revised 10/24/84, 11/14/85; accepted 1/18/85.

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36), some of which are regulated by insulin e.g., proline (31) and glucose (24). Thus, we also investigated insulin binding and its relationship to diet and estrogens in another parameter that may offer insight into the mechanism of dietary lipid enhancement of tumor growth. The data presented here suggest that dietary lipids influence the effects of estrogens on the R3230AC tumor and that the estrogenic milieu as well as insulin binding may play a role in the modification of tumor growth by dietary lipids.

MATERIALS AND METHODS

Diets. The purpose of these studies was to examine the effects of various dietary lipid levels on selected biochemical parameters of the R3230AC mammary tumor. To this end, based on results reported previously (29) and preliminary experiments on tumor growth in intact tumor-bearing rats fed laboratory chow and HF diets, the following experimental protocol was used. Three days prior to tumor implantation, animals were placed on one of the following diets (Table 1): 1) a FF diet containing 0% fat; a LF diet containing 0.5% corn oil; a HF diet containing 20% corn oil; or a HCTO diet containing 20% hydrogenated cottonseed oil (final concentration, <0.1% linoleic acid). All diets, which were offered ad libitum, were devised to contain an equivalent ratio of protein calories to total calories.

Animals, Tumors, and Treatments. Female Fischer rats, 75 to 90 g, were obtained from the Charles River Breeding Laboratory (Wilmington, MA). The R3230AC tumor was implanted s.c. in the axillary region by a sterile trocar technique as described by Hill et al. (28). Bilateral ovariotomies were performed 1 week prior to tumor transplantation, and the tubes were centrifuged for 10 min at 1000 x g, and serum was collected and stored at −76 °C until insulin was measured by radioimmunoassay. The final sucrose concentration was adjusted to 40% (w/w) by appropriate addition of either 66% sucrose or HEPES buffer as determined by a refractometer. The sample was fractionated by flotation through a step gradient containing 25, 33, and 37% sucrose (w/w) at 96,000 x g at 4 °C for 7 h using an SW27 rotor. The material at the 25 to 33% sucrose interface was collected, diluted with 3 volumes of 10 mM Tris-HCl (pH 7.4), and centrifuged for 1 h at 4 °C at 96,000 x g. Membranes were then suspended in a small volume (approximately 1 ml) of 145 mM choline chloride-10 mM Tris-HCl buffer, pH 7.4, for proline studies, or HEPES buffer, pH 7.4, containing 25 mM HEPES, 10 mM NaHCO3, 125 mM NaCl, 3 mM K2HPO4, 1 mM MgSO4, 1 mM CaCl2, and 11 mM glucose with final osmolality of 310 mOsmol, for insulin binding studies, by mixing in a syringe to which a 26-gauge needle was attached. The membrane suspensions were kept at 0 °C until used.

Protein was determined by the method of Lowry et al. (42), and 5'-nucleotidase activity was assayed as described by Morre (43). Plasma membranes were stored at −76 °C until analyzed for fatty acid composition. Lipids were extracted by the method of Foch et al. (20). Extracted lipids were methylated and analyzed by gas-liquid chromatography according to the method of Cave and Erickson-Lucas (12).

Insulin Binding to Plasma Membranes. Crystalline porcine insulin was labeled stoichiometrically with Na125I, using chloramine-T according to the method of Freychet et al. (21). The labeled insulin had a specific activity of 60 to 260 μCi/μg and was greater than 98% trichloroacetic acid precipitable.

The specific binding of 125I-labeled insulin was measured as follows. Ninety to 150 μg of membrane protein were incubated with 125I-labeled insulin in 250 μl of 25 mM HEPES buffer, pH 7.4, containing bovine serum albumin (5 mg/ml) and bacitracin (1 mg/ml) to minimize insulin degradation. Binding of 125I-labeled insulin was performed in triplicate in 1.5-ml conical microfuge tubes at final insulin concentrations of 10−8, 4 x 10−9, 8 x 10−10, 2 x 10−10, 8 x 10−11, and 4 x 10−11 m. For each assay, triplicate samples were incubated in the presence of 10−6 m unlabeled insulin to obtain and then correct for nonspecific binding. The contents of the tubes were mixed, and after a 1-h incubation at room temperature (21 °C), 1 ml of ice-cold 0.9% NaCl solution was added, and the tubes were centrifuged for 4 min in a Beckman 3200 microfuge. Membranes were washed once again with 1 ml of ice-cold 0.9% NaCl solution, and the tubes were drained, blotted, and placed in a Beckman 8000 gamma counter (Beckman Instruments, Inc., Fullerton, CA) for measurement of radioactivity.

Proline Uptake in Plasma Membrane Vesicles. Uptake studies of proline were performed within 2 days of membrane preparation. Samples of 90 to 150 μg of membrane protein, in a volume of 15 μl, were incubated with 15 μl of a solution containing 0.2 mM [3H]proline ([5-3H]-proline, 25 Ci/mmol) in either the choline chloride-Tris buffer (for sodium-independent transport) or 145 mM NaCl-10 mM Tris-HCl buffer, pH 7.4 (for total transport). Final concentrations in the assay were 0.1 mM proline (1 μCi of labeled proline per 30-μl assay) and either 145 mM choline chloride or 72.5 mM NaCl plus 72.5 mM choline chloride. Incubations were done in 1.5-ml microfuge tubes for 20-, 40-, 60-, and 90-s intervals at 21 °C (room temperature), and uptake was stopped by
addition of 1 ml of ice-cold 0.9% NaCl solution. The entire reaction mixture was immediately poured onto Millipore nitrocellulose filters (0.45 μm, 2.5-cm diameter) and washed rapidly with 5 ml of ice-cold 0.9% NaCl solution. Air-dried filters were placed in scintillation vials containing 10 ml of ACS (Amersham), and radioactivity was determined by scintillation counting. Background was determined by the addition of 15 μl of isotope mixture to 1 ml of ice-cold 0.9% NaCl solution containing 15 μl of membrane vesicles.

Insulin Radioimmunoassay. Serum samples were stored at −76 °C until assayed. Insulin levels were measured by means of radioimmunoassay (Radioassay Systems Laboratories, Inc., Carson, CA).

Data Analysis. The data were evaluated statistically by the Student t test; P ≤0.05 was considered to be significant. The Scatchard plots in Chart 1 were analyzed by the Mann-Whitney test.

RESULTS

Influence of Diet on Tumor Burden. The effects of the various diets and hormone treatments on the R3230AC mammary tumor weights and body weights are summarized in Table 2. Tumor burden, which expresses tumor weight per 100-g body weight to account for differences in dietary intake, is also tabulated. The data are presented as separate experimental series since, for each experimental series, animals fed the HF diet were always sacrificed on the same day as an equal number of paired animals fed either the FF, LF, or HCTO diet (3 to 6 animals/week/diet group). It was reported previously (29) that the R3230AC transplanted mammary tumor displayed a more than 2-fold-greater tumor burden in animals fed HF versus FF diets (Series A1) and in animals fed HF versus LF diets (Series B1). These small differences, however, were consistently obtained for tumor gross weight as well as for tumor burden. Animals, which were ovariectomized and placed on these diets, showed more modest differences in tumor weights and no difference in tumor burden when comparisons were made between the HF-fed and the LF- or FF-fed rats.

To ascertain whether alterations in dietary lipids might influence the effects of estrogen treatment on R3230AC tumor growth, ovariectomized tumor-bearing animals were treated with pharmacological doses of EV. The results (Table 2) imply that the efficacy of estrogen treatment may be influenced by the diet ingested, since tumor burden was inhibited to a lesser extent in animals consuming HF diets compared to animals ingesting LF or FF diets. For example, tumor burden was reduced by an average of 28% by estrogen treatment of HF-fed animals (Series A2 versus A3 and B2 versus B3), whereas in LF- (Series B2 versus B3) and FF-fed (Series A2 versus A3) rats, estrogen treatment reduced tumor burden by 51% in each case. An alternative interpretation of these data would suggest that pharmacological doses of estrogens amplify the effects of dietary lipids on tumor growth. Thus, although estrogen therapy is known to inhibit growth of this adenocarcinoma (28), the relative difference between dietary groups, i.e., HF versus FF or HF versus LF, was greater in the presence of high levels of estrogens compared to endogenous (intact) levels, which, in turn, were greater than in the absence of estrogens (ovariectomized animals). Regardless of the interpretation used, the results implicate a relationship between dietary lipids, estrogens, and tumor growth.

The effect of ingestion of an HCTO diet on tumor growth is also presented in Table 2 (Series C1). Tumor burden from animals fed the high-saturated-fat diet, containing <0.1% linoleic acid, was significantly less than tumor burdens from animals fed the HF diet. This suggests that the presence of polyunsaturated lipids is important for the dietary-lipid alteration of R3230AC mammary tumor growth.

Interaction of Dietary Lipid and Estrogen Status on the Characteristics of Insulin Binding. Since dietary lipids caused alterations in tumor growth and these alterations appeared to be expressed in the presence of estrogens, it was of interest to ascertain whether relative tumor growth behavior could be related to insulin status, e.g., insulin receptors. Initial experiments were conducted to examine the effects of dietary lipid ingestion...
on $^{125}$I-insulin binding to plasma membranes of tumors from intact rats fed HF or LF diets. Binding of insulin was measured over a range of $4 \times 10^{-11}$ to $10^{-8}$ M (6 different concentrations). The results from 2 separate experiments (data not shown) indicated that tumor plasma membranes from LF-fed rats displayed $22 \pm 2\%$ less insulin binding than tumor plasma membranes from HF-fed rats; this decrease represents the average decrease obtained by comparison of binding at each insulin concentration studied and displayed a significant difference between HF and LF ($P < 0.01$ by the Mann-Whitney test).

In an effort to maximize the effects of dietary lipids and to study the influence of varying the estrogen status of the tumor-bearing host, animals from 3 different experimental groups, i.e., intact, ovariectomized, or ovariectomized treated with EV, were fed either HF or FF diets. Binding of $^{125}$I-insulin to plasma membranes of tumors from each of these groups was measured over a range of $4 \times 10^{-11}$ to $10^{-8}$ M insulin; the data for specific binding were plotted according to Scatchard (48) and are illustrated in Chart 1. Typical curvilinear plots were obtained for both dietary groups. The results suggest a possible relationship of insulin binding with the tumor growth data based on a greater amount of insulin bound to membranes from intact rats fed HF diets ($P < 0.01$ by the Mann-Whitney test). This difference in insulin binding to tumor membranes from HF- versus FF-fed animals was not observed in lesions from ovariectomized rats (Chart 1B); when ovariectomized animals were treated with EV, the difference in insulin binding attributed to dietary fat was manifested again (Chart 1C, $P < 0.01$ by the Mann-Whitney test). Binding parameters of insulin binding were estimated from the individual Scatchard plots (see the legend to Chart 1). Although estimations of $K_a$ values and number of binding sites from curvilinear plots are subject to error, it would appear that differences in insulin binding observed in membranes from HF-versus FF-fed rats were not due to differences in affinities (neither high nor low affinity sites, assuming a 2-site model, nor in $K_a$ values, assuming a one-site model displaying negative cooperative interactions). The data suggest that the number of binding sites estimated from these plots may be higher in membranes from HF-fed rats. The effect of pharmacological doses of EV to reduce insulin binding was attributable to a reduced number of high affinity sites without an alteration in their $K_a$.

**Influence of Diet on Estrogen-induced Decrease in Insulin Binding to R3230AC Tumor Plasma Membranes.** Studies were performed to determine whether estrogen treatment in vivo would similarly affect insulin binding to tumor plasma membranes of animals ingesting various lipid diets. Animals were ovariectomized and divided into 4 dietary groups, FF, LF, HF, or HCTO; one-half of the animals in each group received estrogen treatment. From the summary of data (Table 3), administration of EV significantly reduced insulin binding to tumor membranes at each concentration of ligand studied (physiological to superphysiological levels), regardless of the diet ingested. For each of the 6 concentrations of insulin examined, the percentage of inhibition resulting from estrogen treatment was computed; these values were then averaged to yield an overall decrease in binding attributable to administration of EV. The latter results show that estrogen treatment reduced insulin binding to a greater extent in tumors from FF- and LF-fed animals compared to HF-fed animals. As was the case for the FF and LF diets, estrogen treatment of HCTO-fed rats reduced insulin binding to a significantly greater extent than that observed for HF-fed rats, suggesting that the magnitude of response to estrogen treatment may be due to the amount of polyunsaturated lipid in the diet. Taken together, these findings indicate that both tumor growth and insulin binding were inhibited to a greater extent by EV treatment in animals fed FF or LF diets.

**Serum Insulin Levels.** Serum insulin levels in the above animals were determined to ascertain whether their alteration could be responsible for the observed differences in insulin binding, i.e., possible down-regulation of the insulin receptor. If this were the case, one would anticipate an inverse relationship between serum insulin levels and insulin binding. The data (Table...
The effects of various diets and estrogen treatments on the proportion of linoleic acid (C 18:2) and arachidonic acid (C 20:4) as well as a significantly lower percentage of oleic acid (C 18:1) than did membrane samples from animals fed FF, LF, or HCTO diets. These differences were maintained regardless of the estrogenic status of the animal. However, administration of EV produced a modest decrease in the relative percentage of C 16:0. The fatty acid distribution profile for the membrane sample from HCTO-fed rats was more similar to that of the FF-fed animals, except for the proportion of linolenic acid (C 18:3), which was lower in the HCTO membrane sample.

Membrane Vesicle Preparations and Proline Uptake. Purified plasma membranes prepared from tumors of animals fed various diets and receiving various hormone treatments were assayed for 5'-nucleotidase activity, in order to assess the similarity of these preparations. The data (not shown) indicate that there was no significant difference in plasma membrane 5'-nucleotidase activities that could be related to diet or hormone treatment, although the activity of this enzyme in membranes from either FF-fed or HCTO-fed estrogen-treated animals appeared to be somewhat lower.

Before experiments to measure proline uptake were performed, the integrity of the plasma membrane vesicles, which readily form from plasma membrane preparations, was determined. Uptake of proline or glucose into vesicles was shown to be sensitive to increasing osmolarity, inhibited by substrates entering by the same carrier, saturable at high substrate concentrations, capable of distinguishing between stereoisomers, and linearly proportional to membrane protein concentration. Electron microscopic examination of plasma membrane preparations displayed vesicle formation with an average diameter of 0.6 μm.

The effects of dietary lipid on the uptake of proline into plasma membrane vesicles are presented in Chart 2. Rats were fed either LF or HF diets, tumors were removed, and plasma membrane vesicles were prepared. Since proline uptake was shown to be stimulated by sodium, the sodium-dependent component of uptake was determined as the difference between uptake in the presence of sodium (total uptake) and uptake in the absence of sodium (sodium-independent uptake). The data also show that the sodium-independent component of uptake was not affected by dietary lipid or estrogen treatment.

Mammary Tumor Plasma Membrane Fatty Acid Composition. The effects of various diets and estrogen treatments on the distribution profile of fatty acids extracted from purified tumor plasma membranes, presented as percentage of total lipid, are summarized in Table 5. Comparisons among various diet groups show several significant differences. Membranes from rats ingesting HF diets had significantly greater percentages of linoleic acid (C 18:2) and arachidonic acid (C 20:4) as well as a significantly lower percentage of oleic acid (C 18:1) than did membrane samples from animals fed FF, LF, or HCTO diets. These differences were maintained regardless of the estrogenic status of the animal. However, administration of EV produced a modest decrease in the relative percentage of C 16:0. The fatty acid distribution profile for the membrane sample from HCTO-fed rats was more similar to that of the FF-fed animals, except for the proportion of linolenic acid (C 18:3), which was lower in the HCTO membrane sample.

DISCUSSION

Unlike the more frequently designed experiments, in which treatment perturbation would be compared to a control (animals

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**Table 3**

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<th>LF</th>
<th>HCTO</th>
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**Table 4**

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**Table 5**

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**Table 6**

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4) reveal that no simple relationship existed between insulin levels and insulin binding to membranes. For example, when comparing insulin binding in HF- versus FF-fed animals (Chart 1), down-regulation of insulin receptors may have occurred. For the HF-fed intact and EV-treated animals, lower serum insulin levels were observed concomitantly with higher insulin binding, and for the ovariectomized animals, no differences in serum insulin levels were accompanied by no differences in insulin binding. However, when examining the effects of estrogen treatment, insulin binding to membranes was lower in estrogen-treated ovariectomized versus untreated ovariectomized rats, yet serum insulin levels were either similar or lower in the estrogen-treated animals. Likewise, reduction of insulin binding resulting from estrogen treatment of HCTO-fed rats was accompanied by lower serum insulin levels. We therefore suggest that alterations in insulin binding due to dietary lipids and estrogen status cannot simply be attributed to down-regulation of the insulin receptor.

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DIETARY LIPIDS AND MAMMARY TUMOR GROWTH

Table 5

Fatty acid composition of tumor plasma membranes

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<thead>
<tr>
<th>Diet</th>
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<th>C 18:2</th>
<th>C 20:4</th>
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<td>1.71 ± 0.11</td>
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<tr>
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<td>16.2 ± 1.0</td>
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<td></td>
<td>Ovariectomized + estradiol</td>
<td>23.6 ± 3.1</td>
<td>15.3 ± 2.3</td>
<td>2.85 ± 0.75</td>
<td>20.8 ± 0.8</td>
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<tr>
<td>FF®</td>
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<td>28.9 ± 0.4</td>
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<tr>
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<td>Ovariectomized</td>
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<td>23.8 ± 2.2</td>
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<td>9.72 ± 1.8</td>
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<tr>
<td></td>
<td>Ovariectomized + estradiol</td>
<td>21.7 ± 1.5</td>
<td>19.8 ± 2.4</td>
<td>—</td>
<td>9.27 ± 0.97</td>
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<tr>
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</tr>
<tr>
<td>LF</td>
<td>Intact</td>
<td>27.7</td>
<td>24.1</td>
<td>—</td>
<td>7.26</td>
</tr>
</tbody>
</table>

*Mean ± SE of 2 separate membrane samples.

—, values below level of detection (<0.02%).

and HF was significant. It was noted previously that, for several transplantable mammary tumors, as little as 0.1% linoleate in the diet resulted in tumor growth equivalent to that seen with 15% corn oil-containing diets (29). Although 0.1% linoleate was not sufficient to alter the growth of the R3230AC tumor relative to a FF diet (29), preliminary work indicated that a 0.5% corn oil diet (~0.3% linoleate) was sufficient. Since the previous report on the R3230AC tumor compared FF to HF-fed rats, and in order to study maximum effects of dietary lipid on alterations of tumor growth, we chose to compare the behavior of tumors from animals fed HF with LF diets or HF with FF or HCTO diets. Over the short term of these experiments, no gross evidence of essential fatty acid deficiency was observed in animals fed FF or HCTO diets, and furthermore, body weights of these animals differed by only 6 to 7% of that attained by HF-fed rats.

The present studies indicate that: (a) the estrogenic environment of the host animal was an important factor in the ability of dietary fats to alter growth of this tumor, (b) dietary lipids influenced the response of this transplantable tumor to estrogen treatment; (c) the host estrogenic environment played a role in the ability of dietary lipids to alter insulin binding; and (d) dietary lipids modulated the decrease in insulin binding resulting from administration of EV.

To study the role of ovarian hormones, tumor growth was examined in both intact and ovariectomized rats. The selection of the R3230AC mammary tumor was predicated on its ability to grow well in ovariectomized hosts (27) and its more rapid growth in animals fed a high polyunsaturated fat diet relative to a FF diet (29). When compared to ovariectomized rats, little or no difference in tumor burden could be attributable to dietary lipids, intact animals or ovariectomized animals treated with EV displayed greater differences in tumor burdens between animals ingesting HF and LF or FF diets. Our use of a transplantable tumor obviates any elucidation of the role of estrogens and dietary fat on tumorigenesis; rather, the experiments reported here suggest a role for estrogens in the dietetic lipid modulation of tumor growth. Cohen et al. (16), based on their studies of DMBA-induced tumors in ovariectomized rats, report that dietary lipid enhanced the incidence of tumors in the absence of ovaries. However, Aylsworth et al. (3) reached a different conclusion from that of Cohen et al., suggesting that a 20% corn oil diet was not sufficient to stimulate tumorigenesis in animals ovariectomized 5

Chart 2. Time course of proline uptake by tumor plasma membrane vesicles prepared from animals fed either HF (Δ) or LF (○) diets. Uptake was measured in either the presence (Total) or absence (Na+ Independent) of 72.5 μM Na+. Na+-dependent uptake was calculated as the difference between total and Na+-independent uptake. Points, mean from 3 separate experiments; bars, SE.

receiving a placebo or sham operation, studies of the influence of dietary lipids on tumor growth have examined the effects of various lipid-containing diets on the alteration of tumor growth. Previous reports have compared high unsaturated fat diets with FF diets (29, 30), 0.5% unsaturated fat diets (12, 38), 5% unsaturated fat diets (chow) (18), or high saturated fat diets (1, 33). Therefore, in the absence of a universally accepted control, any conclusions concerning the effects of dietary lipids must of necessity consider the types of diets that were used.

In the studies presented here, comparisons were made for tumor growth behavior among animals ingesting diets that varied in lipid content and composition. Preliminary observations were obtained by comparing tumor growth in laboratory chow-fed rats to that observed in rats fed HF and LF diets. The tumor weights after approximately 3 weeks provided a relative order of LF < chow < HF, although only the difference in weight between LF and HF was significant. It was noted previously that, for several transplantable mammary tumors, as little as 0.1% linoleate in the diet resulted in tumor growth equivalent to that seen with 15% corn oil-containing diets (29). Although 0.1% linoleate was not sufficient to alter the growth of the R3230AC tumor relative to a FF diet (29), preliminary work indicated that a 0.5% corn oil diet (~0.3% linoleate) was sufficient. Since the previous report on the R3230AC tumor compared FF to HF-fed rats, and in order to study maximum effects of dietary lipid on alterations of tumor growth, we chose to compare the behavior of tumors from animals fed HF with LF diets or HF with FF or HCTO diets. Over the short term of these experiments, no gross evidence of essential fatty acid deficiency was observed in animals fed FF or HCTO diets, and furthermore, body weights of these animals differed by only 6 to 7% of that attained by HF-fed rats.

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Differences in length of experiments, diets, and type of fat studied could account for differences in their conclusions. In either case, the conclusions reached by these investigators were directed primarily towards mammary tumorigenesis rather than growth of mammary tumors. Since it appeared that the estrogenic status of the host played a role in the ability of dietary lipids to alter tumor growth, we examined whether inhibition of tumor growth resulting from estrogen treatment would, in turn, be influenced by dietary lipid intake. The data reported here show that, in ovariectomized animals fed either a LF or FF diet, estrogen-induced inhibition of tumor growth was almost 2-fold greater than in animals fed a HF diet. It would appear, then, that tumors from animals fed the HF diet were more resistant to estrogen treatment or that tumors from animals fed the LF or FF diets were more responsive.

The data from insulin binding studies could offer a possible explanation as to how dietary lipids influence both tumor growth and the effect of estrogens on tumor growth. It seemed curious that, of the various hormones examined as possible mediators of the dietary lipid-altered growth of mammary tumors, little attention had been given to insulin. Insulin is known to be a lipogenic hormone, and further, many of the dietary regimens contain an altered carbohydrate composition concomitant with the altered lipid levels. Since it has been shown that insulin is another hormonal factor involved in growth of the R3230AC tumor (17, 26), we addressed the question of whether insulin binding to tumor plasma membranes was altered by dietary lipids. Ginsburg et al. (23) reported that an increase in the unsaturated fatty acid composition of plasma membranes from Friend erythroleukemia cells resulted in an increase in insulin binding, attributable to an increase in the number of low-affinity binding sites. Grunfeld et al. (24) demonstrated a decrease in insulin binding to 3T3-L1 cells maintained in medium containing saturated fatty acids. When compared to a high-carbohydrate diet, adipocytes or plasma membranes from livers of animals fed HF diets displayed lower insulin binding (37, 53), although Lavau et al. (41) were unable to demonstrate any difference in insulin binding to adipocytes of rats fed HF or LF diets. Results presented here demonstrate that insulin binding to R3230AC plasma membranes from HF-fed animals was greater than binding to membranes from FF- or LF-fed animals. This increased insulin binding was, however, dependent on the estrogen status of the host, as was the alteration of tumor growth.

It was reported previously that estrogen therapy inhibited growth and reduced insulin binding to R3230AC tumors (28, 50). Results reported here indicate that estrogen treatment in vivo inhibited insulin binding to tumor plasma membranes regardless of the level of dietary lipid ingested. However, inhibition of insulin binding by estrogen treatment was more pronounced in tumor membranes from animals fed low levels of unsaturated lipids, i.e., FF, LF, and HCTO diets. These results indicate that dietary lipids influence, in a similar manner, the efficacy of EV treatment to reduce both tumor growth and insulin binding. Although these results do not prove a cause-and-effect relationship, they do suggest a role for insulin in the mediation of the dietary lipid effects on growth of this tumor.

We next addressed the question of how insulin binding may have been affected by dietary lipids. Serum insulin levels, plasma membrane fatty acid composition, and plasma membrane 5'-nucleotidase activities were determined to ascertain their possible relationship to the dietary lipid-induced alteration in insulin binding. 5'-Nucleotidase activities, which would reflect the relative purity of the membrane preparations, were comparable among the various experimental groups and, thus, would not likely account for the observed differences in insulin binding. Dietary lipids were shown to alter the fatty acid composition of the tumor membrane preparations, possibly accounting for differences in hormone binding. However, these differences in fatty acid composition between membranes from HF- and FF-fed animals were also seen in membrane samples from ovariectomized animals, where no difference in insulin binding was observed. Differences in serum insulin levels due to various diets and/or hormone treatments could lead to alterations in insulin binding capacity, i.e., down- or up-regulation of the receptors (6, 8, 15, 40, 44). No uniform picture has emerged regarding the effects of ovariectomy and/or administration of estrogens on serum insulin levels. In non-tumor-bearing rats, ovariectomy lowered and estradiol treatment elevated serum insulin (5, 49, 54), whereas in mammary tumor-bearing rats, no significant alterations in serum insulin resulted from ovariectomy or administration of estrogens (50, 51). It should be noted that, in all of these studies, animals were fed laboratory chow diets which contain primarily complex carbohydrates, whereas the diets utilized here contain both simple and complex carbohydrates. In the studies reported here, no consistent relationship between serum insulin levels and insulin binding capacity was obtained to suggest down- or up-regulation of the insulin receptor. Both Salans et al. (47) and Ip et al. (37) had earlier reported no direct relationship between serum insulin levels and insulin binding to rat adipocytes from animals consuming HF and LF diets. A relationship between serum insulin levels and tumor growth might be suggested, since serum insulin levels were higher in FF-fed rats compared to HF-fed animals, as also reported by others studying various diets (2, 9, 45). Growth of the R3230AC tumor was reported to be inhibited by administration of insulin (17), and in these studies, tumor growth was slower in FF-fed rats. This simplistic explanation would not be borne out, however, by the data obtained from HCTO-fed animals, whose tumors grew slower than those of HF-fed rats despite comparable serum insulin levels. Apparently, factors other than the above must be responsible for the results observed here.

The question of whether alterations in membrane composition by dietary lipids can affect substrate uptake was examined. It has been proposed that increased nutrient uptake resulting from changes in the cell membrane may be a primary cause of malignant growth (32). Kaduce et al. (39) reported that a polyunsaturated lipid diet, relative to a saturated lipid diet, led to increased levels of plasma membrane polyenoic fatty acids in Ehrlich ascites cells along with increased sodium-dependent αAIB uptake. 3T3-L1 cells exposed to saturated fatty acids in culture exhibited up to an 80% decrease in insulin-stimulated 2-deoxyglucose uptake (24). The data presented here show that, although plasma membrane fatty acid composition was affected by dietary lipids, this modification in membrane composition was not accompanied by an alteration in the uptake of proline. These data, however, do not preclude the possibility that proline transport in vivo may have been affected by dietary lipids.

In summary, we have demonstrated a relationship between dietary lipid alteration of tumor growth, host estrogen status,
and insulin binding. Altered tumor growth due to ingestion of high levels of unsaturated lipids was more apparent in both intact and EV-treated ovariectomized rats than in ovariectomized rats; this altered tumor growth was accompanied by a similar alteration in insulin binding capacity of the tumor plasma membranes. Inhibition of tumor growth by estrogen treatment was greatest in animals fed low unsaturated lipid diets, and likewise, inhibition of insulin binding by estrogen treatment was greatest in tumors from females fed these diets. These data suggest a possible role for estrogens as well as for insulin binding in the mediation of the dietary lipid-altered growth of this tumor, although the mechanism by which altered insulin binding might affect tumor growth remains to be shown. These data also have implications for the therapy of breast cancer, inferring that the ingestion of a LF diet may enhance the efficacy of estrogen treatment.

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