Requirement of Essential Fatty Acid for Mammary Tumorigenesis in the Rat¹

Clement Ip,² Christopher A. Carter, and Margot M. Ip

Department of Breast Surgery [C. I., C. A. C.) and Grace Cancer Drug Center [M. M. I.], Roswell Park Memorial Institute, Buffalo, New York 14263

ABSTRACT

In an attempt to determine the requirement of essential fatty acid for dimethylnitrobenz(a)anthracene-induced mammary tumorigenesis, rats were fed diets containing different levels of linoleate: 0.5, 1.1, 1.7, 2.2, 3.5, 4.4, 8.5, or 11.5%. Each diet contained 20% of fat by weight, with varying amounts of coconut oil and corn oil added to achieve the desired levels of linoleate. Mammary tumorigenesis was very sensitive to linoleate intake and increased proportionately in the range of 0.5 to 4.4% of dietary linoleate. Regression analysis indicated that a breakpoint occurred at 4.4%, beyond which there was a very poor linear relationship, suggesting the possibility of a plateau. From the intersection of the regression lines in both the upper and lower ranges, the level of linoleate required to elicit the maximal tumorigenic response was estimated to be around 4%. The differences in tumor yield could not be correlated with changes in prostaglandin E concentration in the mammary fat pads of normal animals maintained on similar diets, suggesting that linoleate may act by some other mechanism to stimulate mammary tumorigenesis.

INTRODUCTION

Diets rich in fat are known to enhance the development of tumors in several rodent mammary cancer models. Most of the studies involve chemically induced mammary tumors in rats, although there are a number of reports which show that dietary fat is also capable of increasing the growth of both spontaneous and transplantable mammary tumors in mice. An excellent review of this subject by Welsch and Aylsworth (22) has appeared recently in the literature. In addition to quantity of fat, the type of fat has also been considered to be important. Carroll and Khor (4) assessed the effect of 10 different fats on the occurrence of DMBA²-induced mammary tumors in rats, and found that in general, polyunsaturated fat diets gave a higher tumor yield than did saturated fat diets. Similar findings have been reported by other investigators (7, 10, 13–15, 19), including our own laboratory (11).

In further studies, Carroll and Hopkins (3) demonstrated that diets containing 3% sunflower seed oil (polyunsaturated fat) and 17% beef tallow or coconut oil (saturated fats) enhanced tumorigenesis as much as did a diet containing 20% sunflower seed oil. Rats on these diets developed twice as many tumors as those fed diets containing 20% of the saturated fats alone. These observations suggest that there may be a requirement for polyunsaturated fat in mammary tumorigenesis, which is not satisfied by fats such as coconut oil or beef tallow, but can be provided by adding 3% sunflower seed oil to diets containing these fats. A recent paper by Cave and Jurkowski (6) also showed that when the polyunsaturated lipid content (corn oil) of the diet fell below 3% there was a decrease in tumor incidence in rats treated with N-methyl-N-nitrosourea. It is thought that linoleate may be the essential fatty acid primarily responsible for the tumor-promoting effect of unsaturated fat (14).

The objective of the present study was to test the hypothesis that there is a minimal requirement of EFA for mammary tumorigenesis, and to delineate what this requirement is. In the text, the terms linoleate and EFA are used interchangeably, since from a nutritional standpoint, as well as from the basis of our experimental design, linoleate is the major source of EFA in the diet. The relationship between dietary linoleate intake and tissue prostaglandin content was also explored.

MATERIALS AND METHODS

Animals and Diets. Female Sprague-Dawley [Crl:CD(SD)BR] rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) at 40 days of age. They were housed in a temperature- and light-controlled room with food and water available ad libitum. All animals were fed Purina laboratory chow pellets until 3 days after carcinogen administration, at which point they were divided into groups of 30 rats each, according to the synthetic diet given. The diets contained 20% of fat by weight, with each varying in the composition of fats blended to achieve different levels of linoleate. All other ingredients, namely casein, dextrose, vitamin and salt mixes, and Alphacel, were held constant. The composition of the 20% fat synthetic diet has been described in detail in a previous publication (17).

In Experiment 1, the fat blends consisted of a mixture of palm oil and corn oil in different proportions, such that when added at 20% by weight to the diet, it yielded increasing levels of linoleate (EFA): 2.0, 4.1, 6.5, or 12.1%. In Experiment 2, the fat blends consisted of a mixture of coconut oil and corn oil to give a wide range of EFA in the diet: 0.5, 1.1, 1.7, 2.2, 3.5, 4.4, 8.5, or 11.5%. The actual EFA content was confirmed by the lipoxidase assay (20), which measures cis,cis-methylene interrupted double bonds, as in linoleic acid. The different diets were started 3 days after DMBA intubation, and were continued until termination of the experiment.

Tumor Induction. Mammary tumors were induced by intragastric administration of 5 mg of DMBA at 50 days of age. The method of DMBA administration has been described previously (17). In Experiment 2, all rats received a second 5-mg dose of DMBA 10 weeks after the first. The reason for this protocol is described in detail in "Results." Rats were palpated weekly to determine the appearance and location of the tumors. Approximately 15 to 30% of the animals in each group had to be sacrificed before termination of the experiment, when they became moribund on account of increased tumor burden. Otherwise, the decision was made to terminate the study when the incidence curves started to level off. At autopsy, animals were examined for nonpalpable tumors. All tumors were excised, fixed in buffered formalin, and sectioned for histology. Mammary tumor pathology was confirmed according to the criteria of Young and Hallowes (24). Only adenocarcinomas are reported in "Results."

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² To whom requests for reprints should be addressed.

¹ The abbreviations used are: DMBA, 7,12-dimethylnitrobenz(a)anthracene; EFA, essential fatty acid (linoleate); PGE, prostaglandin E.

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Tissue Prostaglandin Determination. Animals were fed the different EFA diets as in Experiment 2 for 1 month, starting at 50 days of age. They were killed by cervical dislocation, all on the same day in the morning, in order to avoid the effect of prolactin surge in the afternoon of proestrus. These animals were not treated with DMBA, and no attempt was made to identify the stage of the estrous cycle when they were killed. The mammary fat pad was excised at the time of sacrifice and frozen in liquid nitrogen. PGE was extracted and assayed according to slight modifications of published procedures (16, 21). After pulverization, the tissue sample (0.5 g) was homogenized in a Potter-Elvehjem homogenizer in 2.5 ml of 0.1 M Tris-HCl buffer (pH 7.4), containing 0.15 M sodium azide. Upon removal of the fat layer and cell debris after low-speed centrifugation, the supernatant was subjected to a second centrifugation at 100,000 x g for 1 h. This high-speed supernatant was acidified to pH 3.5 with 1 to 2 drops of 1 M citric acid and a tracer amount of tritiated PGE{sub 2} \((5,8,8,11,12,14,15-{\text{H}})\text{PGE}{_2}\); New England Nuclear, Boston, MA) was added. A 300-\(\mu\)l aliquot of this sample was then extracted with 900 \(\mu\)l of ethyl acetate. After separation by centrifugation, the organic layer was removed by snap freezing the inorganic layer and decanting the organic layer into a fresh test tube. This was followed by evaporating the solvent under \(N_2\) and resuspending the residue in the original volume of buffer. Recovery was typically between 65 and 85%. Determination of PGE was carried out using anti-PGE antibody obtained from Seragen (Boston, MA). Cold PG{sub E} from Cayman Chemical (Ann Arbor, Mi) was used as the standard. The lowest detectable level of PGE in this assay is 10 pg; the cross-reactivity of the antibody with PGE{sub E} is 100%.

Statistical Analysis. The tumor data from the different dietary groups were analyzed as a set in relation to their EFA intake by the linear regression method (18). The statistical analysis is described in detail in "Results."

RESULTS

The effect of 4 dietary EFA levels on mammary tumorigenesis in rats fed a 20% fat diet was examined in a preliminary experiment (Experiment 1). As can be seen from the tumor incidence curve of Chart 1, the risk of a rat developing a tumor appears to increase with increasing intake of EFA. A similar conclusion can be drawn from Chart 2, in which the total number of palpable tumors at each time point is plotted, although in this case, the group fed 4.1% EFA overlapped with the 12.1% EFA group at the later time periods after DMBA. This experiment therefore suggested that there was an EFA requirement for mammary tumorigenesis, since in rats fed a high fat diet there was a marked difference in tumorigenesis between the lowest and highest EFA groups. Furthermore, because of the overlap among the 3 highest EFA groups, the data suggested that there might possibly be a break point above 4%, and that a further experiment, with a wider range of EFA levels, would be necessary in order to titrate more precisely the EFA requirement.

A second experiment was therefore carried out, using 8 different levels of EFA, ranging from 0.5 to 11.5% in the diet, in an attempt to delineate more precisely the shape of the tumorigenic response curve. Before presenting the results of this study, we would like to discuss an unusual feature that developed during the course of the experiment. Based on several years of experience with this tumor model, we have consistently observed that in rats fed a 20% corn oil diet (approximately 12% EFA), the tumor incidence was about 30 to 40% by 10 weeks after p.o. administration of 5 mg DMBA. Thus, we became increasingly concerned as the study progressed, when our record indicated that the incidence was extremely low in the first half of the experimental period. At 10 weeks after DMBA, there was only one tumor-bearing rat in each group, with the exception of the 0.5 and 1.5% EFA groups, in which there was none. It was decided at this point to administer another 5-mg dose of DMBA in an attempt to increase the tumor yield. Whether it was due to the boost from the second carcinogenic insult or to an abnormally long latent period, a more distinctive pattern finally emerged by Week 15, showing an enhancement of tumor development with increasing levels of EFA in the diet. Since the objective of the present study was not to determine whether dietary fat affects the initiation or promotion stage of carcinogenesis, but rather if the tumorigenic response depended on the EFA intake, the introduction of a second dose of DMBA should not compromise the interpretation of the results. The incidence and tumor yield curves are shown in Charts 3 and 4, respectively. In view of the low tumor incidence with the resultant clustering among the groups before Week 15, the time course of tumor development is illustrated starting from this time point up to termination of the experiment at Week 25.

From the data, it appears that mammary tumor development increased in a step-wise manner with increasing EFA levels in.
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Chart 3. Incidence of palpable mammary tumors in rats fed diets containing different levels of EFA (Experiment 2). There were 30 rats/group.

Chart 4. Cumulative number of palpable mammary tumors in rats fed diets containing different levels of EFA (Experiment 2). There were 30 rats/group.

The data from Weeks 15 through 20 were inconclusive in supporting any claim to a breakpoint. For the last 5 weeks (from Week 20 through autopsy), a pattern became apparent in the sense that a strong linear relationship exists between EFA and the tumorigenic response. This observation is supported by the high $R^2$ value associated with the range. A summary of the regression analysis for the autopsy data corresponding to those in Chart 5 is shown in Table 1. The $R^2$ values exceed 0.9 for all the ranges up to and including 0.5 to 4.4%. For EFA ranging from 0.5 to 8.5%, the $R^2$ value drops off dramatically, compared to the $R^2$ value for the previous range of 0.5 to 4.4%, leading to the assumption that a change in slope occurs around this point. The data in the range of 4.4 to 11.5% exhibit a poor linear relationship ($R^2 = 0.059$ for the incidence data), indicating a possible plateau.

Further analysis was carried out to determine an estimate of the breakpoint. For each response, i.e., the number of rats with tumors and the total number of tumors (out of a total of 30 rats), regression equations were computed for the ranges of 0.5 to 4.4% EFA and 4.4 to 11.5% EFA, using the autopsy data shown in Chart 5. These equations are presented in the lower half of Table 1. The percentage of EFA at which the lines intersect is...
the breakpoint estimate. According to this procedure, the breakpoint was found to be 4.2% EFA for the incidence response, and 4.1% EFA for the tumor yield response.

The growth curves of rats ingesting the different EFA diets in Experiment 2 are shown in Chart 6. The shaded area between the 2 lines represents the range of the average body weights for the 8 dietary groups. The reason for presenting the data in this manner is because of the very small differences among the groups, which results in too much overlap to clearly distinguish the individual lines. This was especially true for the first 10 weeks of the experiment. For the next 15 weeks, a pattern seemed to emerge in which rats fed the 0.5 and 1.1% EFA diets were gaining weight at a slightly slower rate than the rest. This discrepancy is reflected by the wider dispersion of the shaded area. At the time of sacrifice, the final carcass weights for the 0.5, 1.1, 1.7, 2.2, 3.5, 4.4, 8.5, and 11.5% EFA groups were as follows: 331 ± 7 (SE), 332 ± 6, 351 ± 5, 351 ± 6, 358 ± 8, 345 ± 6, 352 ± 8, and 347 ± 4 g, respectively. Thus, it is unlikely that the difference in weight gain could account for the gradation in tumor yield of these rats.

Since linoleate serves as the precursor for prostaglandin synthesis, it seemed reasonable to determine whether tissue prostaglandin concentrations were influenced by increasing intake of linoleate. Animals were fed the 8 different EFA diets for 1 month before sacrifice, and the PGE levels in the mammary fat pad were examined. Results are shown in Table 2. Surprisingly, we found that tissue PGE levels were essentially insensitive to EFA intake, even at the lower range of the titration curve, in which susceptibility to cancer risk was most responsive.

**DISCUSSION**

Results of the present study confirm the conclusion of Carroll and Hopkins (3) that there is a requirement of EFA for the maximal expression of mammary neoplasia. According to our experiment with the DMBA-induced tumor model in the rat, this requirement was found to be approximately 4%. Using the same model, Hopkins et al. (9) have also found that rats fed a high saturated fat diet containing 3% ethyl linoleate developed as many tumors as did those fed a 20% sunflower oil diet, although the latter contained about 4 times as much linoleate. Thus, there is agreement between the 2 studies on the concept of a saturating level of linoleate in promoting mammary tumorigenesis. In light of this finding, it would appear that previous reports in the literature evaluating the effect of saturated fats have used diets that contained levels of EFA less than that required to elicit the maximal response. This would explain the artifactual observation that polyunsaturated fats are more effective than saturated fats in the enhancement of cancer development.

Human breast cancer mortality shows a strong positive correlation with total fat intake and little or no correlation with vegetal fat intake (2). In countries where fat intake is low, it is most likely that edible fats consist of predominantly polyunsaturates from plant sources. However, in countries where fat intake is high, a greater proportion tends to be saturated fat from animal sources. Unfortunately, epidemiology data cannot produce the information of what the EFA requirement is for human breast cancer. Assuming such a requirement exists and is relatively low, most human diets are likely to supply enough EFA to permit an optimal tumor response. If this is the case, the conclusion of our study would help to explain why differences in the relative ability of unsaturated versus saturated fats to promote tumor development in the animal model are less apparent at high fat intake. Once the EFA requirement for optimal tumor expression is met, further enhancement of development would depend on the amount and not on the type of dietary fat.

The role of linoleate in regulating tumor development has not yet been elucidated. However, it is known that linoleate, via the intermediate arachidonic acid, can serve as the precursor of the prostaglandins and related compounds of both the cyclooxygenase and lipoxygenase pathways. Furthermore, we have recently shown that indomethacin, an inhibitor of prostaglandin synthetase, inhibits the stimulatory effect of fat on mammary tumorigenesis (5). While the role of the prostaglandins is complex and
not well understood, it is thought that they play an important role in tumorigenesis, tumor proliferation, and metastasis (8).

The lack of a correlation between mammary gland PGE levels and dietary EFA intake observed in this study does not necessarily mean that linoleate does not affect tumorigenesis via stimulation of prostaglandin synthesis. However, it does indicate that the interaction is probably far more complex than originally envisioned. Moreover, several interpretations of the data are possible. Thus, prostaglandin production by components of the immune system, rather than by the mammary fat pad, may affect the tumorigenic response. Alternatively, even though tissue levels of PGE were unaffected by dietary fat intake, it is possible that other cyclooxygenase products such as prostacyclin or thromboxane, or lipoxygenase products, were affected. It is also possible that prostaglandin synthesis in the mammary fat pad is very insensitive to the bioavailability of linoleate, and that local prostaglandin synthesis is not a critical event in controlling neoplastic expression. Finally, the antibody used in the radioimmunoassay exhibited 100% cross-reactivity with PGE1 and PGE2. If there was a decrease in PGE1 synthesis concomitant with an increase in PGE2 synthesis as dietary EFA increased, it could not be demonstrated in this assay.

Kidwell et al. (12) and Wicha et al. (23) have reported interesting data, showing that polyunsaturated fatty acids can influence the growth of normal rat mammary gland, as well as DMBA-induced mammary carcinoma in cell culture. These investigators found that supplementation of a hormone-enriched medium with linoleic acid increased thymidine incorporation and reduced cell-doubling time. Saturated fatty acids, such as stearic acid, had the opposite effect, thus inhibiting mitotic activity of these cells. A recent paper by Aylsworth et al. (1) suggests that polyunsaturated fatty acids could promote tumorigenesis by inhibition of intercellular communication. While the present study does not address the mechanism by which linoleate acts to enhance neoplastic development, it does reinforce the message that any working hypothesis should be consistent with the in vivo data that there is a linoleate (EFA) requirement for the maximal expression of mammary tumorigenesis.

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