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Review Article

REVIEW OF THE EFFECTS OF DIETARY FAT ON EXPERIMENTAL MAMMARY GLAND TUMORIGENESIS: ROLE OF LIPID PEROXIDATION

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Abstract—The purpose of this communication is threefold, that is, (1) to review and critique the studies designed to examine the interrelationship between dietary fat and experimental rodent mammary gland tumorigenesis, (2) to assess the influence of dietary fat on growth of human breast carcinoma transplants in immunodeficient mice, and (3) to examine and discuss the role of products of lipid peroxidation in these tumorigenic processes. It is concluded from this review and critique that the amount and type of dietary fat can significantly influence the development and/or growth of rodent mammary gland tumors and growth of human breast carcinomas in immune deficient mice. Dietary fat can be either stimulatory or inhibitory to these tumorigenic processes, phenomena that could be a function, at least in part, of the generation of products of lipid peroxidation.

Keywords—Mammary gland tumors, Breast carcinomas, Dietary fat, Lipid peroxidation, Free radicals

INTRODUCTION

Numerous international population correlation studies report a strong positive linear relationship between dietary fat consumption and human breast cancer incidence and/or mortality.[1,2] Thus, among countries in which breast cancer rates vary more than fivefold and in which fat consumption per capita varies widely, those countries with the highest rates of breast cancer are countries with a relatively high estimated fat consumption. Although cohort and case-control studies designed to examine the dietary fat–human breast cancer relationship have been inconsistent,[3–6] the striking positive correlation between dietary fat consumption and breast cancer incidence and/or mortality demonstrated in international population studies provides sufficient impetus to examine this relationship thoroughly in well-controlled experimental animal studies.

The purpose of this communication is to review and critique the dietary fat–mammary gland tumorigenesis relationship in experimental animals. In particular, five issues are examined: (1) amount of fat and rodent mammary gland tumorigenesis, (2) type of fat and rodent mammary gland tumorigenesis, (3) role of calories in rodent mammary gland tumorigenesis, (4) influence of fat on growth of human breast carcinomas maintained in immune deficient mice, and (5) role of products of lipid peroxidation in the modulation of mammary gland tumorigenesis by dietary fat.

The amount of dietary fat and the development of mammary tumors in experimental animals

The amount of dietary fat has a profound effect on the development of mammary tumors in mice and rats. Increased amounts of ingested fat have been reported

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to increase the development of mammary tumors in an array of mouse and rat experimental animal models, for example, "spontaneous" mammary tumors,[7-12] induced (chemical carcinogens, irradiation, hormones) mammary tumors,[13-19] transplantable mammary tumors,[20-22] and metastatic mammary tumors.[22-24] The period (initiation stage, promotion stage, prenatal, prepuberty, postpuberty, young, old, etc.) when hyperalimentation with fat influences this tumorigenic process has been the focus of many laboratories. The carcinogen-induced rat mammary tumor model has been most useful in assessing whether or not dietary fat acts at the initiation stage or the promotion stage of mammary gland tumorigenesis.[25] In this model, the initiation stage is defined as the transformation of a normal mammary ductal cell into a tumor cell by the administration of a carcinogen (chemical or physical); test diets are fed commencing several weeks before carcinogen treatment and continuously until about 1–5 days after carcinogen treatment. The promotion stage is defined as the time period commencing shortly after carcinogen administration and characterizes growth of tumorigenic mammary cells. Increasing the quantity of ingested fats generally does not appear to have a significant effect on the initiation stage of mammary gland tumorigenesis,[26-32] although there are reports to the contrary.[33-35] Thus, profound and consistent effects of increased quantities of dietary fat on the initiation stage of mammary gland tumorigenesis have not been demonstrated. In contrast, increased quantities of fat fed during the promotion stage show consistent increases in the development of mammary tumors.[13-18,27,28,32,36-63] The enhanced development of transplantable mammary tumors in rodents by increasing dietary fat further supports the concept that fat acts during the promotion stage.[20-22] The enhancing effect of increased amounts of dietary fat on the promotion stage is most often observed when the fat is fed commencing early in this stage, usually in young adult animals and shortly after carcinogen treatment. However, increased quantities of fat can also enhance mammary gland tumorigenesis when fed commencing at late time points during the promotion stage (in older animals) and commencing months after carcinogen treatment,[27,29,37,42,48,62,64] Thus, hyperalimentation of fat commencing at either early or late time points during the promotion stage can significantly enhance the development of mammary tumors in rats treated with chemical carcinogens. Conversely, it can be inferred that reducing the amount of fat consumed, even late in the promotion stage (late in an animal's life), can significantly suppress mammary gland tumorigenesis. That high-fat diets can increase the development of "spontaneously" occurring mammary tumors in mice when fed solely during the prenatal period has been reported by a single laboratory[65]; this interesting and potentially important observation, however, has not been confirmed by others.[11] Thus, feeding of high levels of fat solely during the perinatal-puberty or puberty periods did not significantly influence the incidence of "spontaneously" developing mammary tumors in mice when compared to mice fed high levels of fat solely during the postpuberty period.[11] In this latter study, the development of mammary tumors was dependent upon the duration of feeding high levels of fat, not the period in the animal's life in which the fat was fed. Indeed, such a relationship is also observed in rats treated with carcinogens, that is, the length of time that high-fat diets are fed appears to be more important than specific time periods (during promotion) in which these diets are fed.[29,40]

It has been suggested in a number of reports that once a critical level of fat is reached in a diet, a further increase in fat does not result in a parallel increase in mammary tumor development. For example, doubling the fat content of the diet from 10% to 20% (all dietary fat or fatty acid percentages in this communication are by weight) did not result in a significant increase in the development of mammary tumors in carcinogen-treated rats.[51,57,66,67] Stated differently, reducing the fat content by one half (20% to 10%) did not result in a significant reduction in mammary tumor development. These results would suggest that a threshold level for dietary fat (e.g., 10%) does exist; further increases in the level of fat do not result in a parallel increase in mammary tumor development. However, if the level of linoleic acid is maintained at a constant level (4.8%) in a series of diets with wide ranging fat levels, a proportionate enhancement in mammary tumor development (in carcinogen treated rats) is observed with increasing levels of dietary fat (8% to 20%).[15] Thus, at least within this range of dietary fat levels, and constant levels of linoleic acid, a threshold level of dietary fat may not exist, that is, any increase in fat consumption may result in a parallel increase in mammary gland tumorigenesis. An absence of a threshold level of dietary fat in the range of 5% to 20% and mammary gland tumorigenesis also was suggested by other studies.[44,51,57,60] In contrast, a more recent report provides evidence that a threshold level of fat does exist, even when maintaining constant levels of linoleic acid (3.3%), that is, the development of "spontaneous" mammary tumors in mice was increased when fed fat diets of 5% to 11%; from 11% to 20% no further increase in mammary tumor development was observed.[68] Thus, whether or not a threshold level of dietary fat exists in the 5% to 20% range has not yet been unequivocally determined.

The type of dietary fat and the development of mammary tumors in experimental animals

Few laboratories have examined whether or not the type of fat affects the initiation stage of mammary gland tumorigenesis. High dietary levels of saturated fatty acids compared with high dietary levels of polyunsaturated fatty acids fed during the initiation stage have been reported to stimulate mammary tumor development.[31,33,69] Such observations, however, have not been confirmed by others.[30] Considering the small number and inconsistencies of these studies, definitive conclusions at this time are not possible.

In contrast, many studies have shown that the type of dietary fat affects the promotion stage of mammary gland tumorigenesis. Generally high levels of saturated fatty acids from animals (i.e., lard and beef tallow) or plants (i.e., coconut oil and palm oil) suppress the development of mammary tumors compared with high levels of polyunsaturated fatty acids from vegetable oils.[13,46,51-53,63,69-74] Supplementing saturated fatty acids that are low in essential fatty acids, with a small amount of polyunsaturated fatty acids (i.e., linoleic acid) often reverses the mammary tumor development suppression of such fats.[13,63] Thus, mammary tumor development is as high in rats fed a diet containing 20% sunflower-seed oil as in rats fed a diet containing 17% beef tallow and 3% sunflower-seed oil[13] or 17% coconut oil and 3% ethyl linoleate.[63] When these studies were repeated, however, supplementing the saturated fatty acid diet with a similar amount of polyunsaturated fatty acids only partially reversed the mammary tumor suppression of the saturated fatty acid diet.[51] The level of linoleic acid in a diet high in saturated fatty acids and low in polyunsaturated fatty acids had a major influence on the development of mammary tumors; when the level of linoleic acid reached 4.4%, a further increase in the ratio of saturated to polyunsaturated fatty acids did not reduce mammary tumor development.[46] All studies cited above specifically examined the promotion stage of rat mammary gland tumorigenesis.

Other studies also showed an inhibitory effect of high levels of saturated fatty acids, compared with polyunsaturated fatty acids, on rat mammary gland tumorigenesis.[67,75-83] In these studies, the test diets were administered continuously during both the initiation and promotion stages and therefore are difficult to interpret. Studies examining other rodent mammary tumor models also provide evidence that high dietary levels of saturated fatty acids, compared to diets rich in polyunsaturated fatty acids, suppress mammary tumor development. This was demonstrated in "spontaneously" derived[84-88] and transplantable mammary tumors[89-96] in mice and rats. Again, supplementing the saturated fatty acid diets with small amounts of polyunsaturated fatty acids often negates the mammary tumor suppression of the saturated fatty acid diets.[87,92] High-fat diets formulated to suppress cardiovascular disease in humans by having the calories supplied equally by saturated, monounsaturated, and polyunsaturated fatty acids stimulate mammary tumor development in rats to a degree comparable to high-fat diets that mimic those consumed by Western populations, (46% saturated, 38% monounsaturated, and 16% polyunsaturated fatty acids).[97]

Results of studies on the influence of diets rich in monoenoic fatty acids (e.g., oleic acid as found in olive oil and palm oil) on the development of mammary tumors in rodents have been inconsistent.[53,66,67,73,85,87,98-102] In utilizing the carcinogen-induced rat mammary tumor model, the feeding of high dietary levels of olive oil (79% oleic acid) was reported to have no effect[67] or an inhibitory effect[53,102] on the development of mammary tumors. The latter studies examined the promotion stage; the former used a continuous initiation-promotion protocol. Rats fed high dietary levels of an oleic acid-rich mutant safflower-seed oil (77% oleic acid) during the promotion stage developed nearly the same number[98] or increased numbers[102] of carcinogen-induced mammary tumors as rats fed high levels of a standard diet relatively low in oleic acid containing safflower-seed oil (12% oleic acid). A positive association between oleic acid consumption and the enhancement of rat mammary tumorigenesis also was reported.[75] In contrast, rats fed high levels of palm oil (43% oleic acid) during the promotion stage had fewer mammary tumors than did rats fed corn oil (37% oleic acid) or soybean oil (21% oleic acid).[73] The ingestion of high levels of olive oil by mice has been reported to inhibit[85] or to have no effect[87] on the development of "spontaneous" mammary tumors. The development of transplantable mouse[100] and rat[101] mammary tumors was reported not to be influenced by the consumption of diets rich in olive oil. Thus, a consistent inhibitory activity of diets rich in monoenoic fatty acids on the development of mammary tumors in rodents has not been shown. Diets rich in olive oil or palm oil may not contain levels of linoleic acid sufficient for optimal mammary tumor development. Supplementation of olive oil diets with linoleic acid has been reported to block the inhibitory activities of an olive oil diet in rats treated with chemical carcinogens.[102] The reported suppression of mammary tumorigenesis by these oils may not be a function of monoenoic fatty acid levels but merely inadequate linoleic acid content.

Oil from seeds of the evening primrose contains about 75% linoleic acid. It also contains relatively high levels (9%) of γ-linolenic acid, a fatty acid that is rapidly elongated to dihomo-γ-linolenic acid. Feeding diets containing evening primrose oil was reported to inhibit the development of carcinogen-induced rat mammary tumors (promotion stage),[103,104] carcinogen-induced mouse mammary tumors,[105]and transplantable rat mammary tumors.[106,107] This inhibition did not appear to be dose-dependent.[106,107] Although there are few reports showing chemoprevention of mammary tumorigenesis by this oil, these interesting results justify further studies.

In recent years there has been appreciable interest in the chemoprevention of mammary gland tumorigenesis by diets rich in fish oils. Oils from cold-water fish are often rich in omega-3 long chain polyunsaturated fatty acids, the most abundant being eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Mice or rats fed relatively high dietary levels of these oils often show inhibition of mammary gland tumorigenesis. This was observed in the carcinogen-induced rat mammary tumor model during the promotion stage[44,51,104,108] and with a continuous initiation-promotion protocol.[109] Inhibition of mammary tumorigenesis by dietary fish oil also was observed with the carcinogen-induced mouse mammary tumor model,[105] with transplantable mammary tumors in mice[92] and rats,[110-112] and with the "spontaneous" mouse mammary tumor model.[85] Fish oils are often deficient or marginal in linoleic acid; unfortunately, the fish oil diets were not supplemented with this essential fatty acid in a number of these studies. Supplementation with modest amounts of corn oil (e.g., 75% fish oil and 25% corn oil) was reported to block,[92] or partially block,[108] the inhibition of mammary tumor developmental growth by a fish oil diet. High-fat diets rich in fish oil and supplemented with vegetable oils (79% fish oil and 21% corn oil, olive oil, or linseed oil) failed to influence the development of transplantable rat mammary tumors when fed from the day of tumor cell inoculation.[112] A dose response to fish oil diets is indicated in one study[109] but not in several others.[44,51,92,107]

Although it is clear that fish oil diets high in omega-3 long chain polyunsaturated fatty acids (i.e., EPA and DHA) can inhibit mammary gland tumorigenesis in experimental animals, considerably less is known of the effect of shorter chain omega-3 polyunsaturated fatty acids, for example, α-linolenic acid, in this tumorigenic process. Mice fed a diet in which the fat content was derived solely from perilla oil (58% α-linolenic acid) had significantly fewer "spontaneous" mammary tumors than did mice fed a high linoleate safflower-seed oil diet.[113] Mammary gland tumorigenesis in the perilla oil fed mice was also lower than that in soybean oil (18% α-linolenic acid) fed controls.[113] In carcinogen-treated mice, mammary gland tumorigenesis was significantly suppressed in mice fed linseed oil (47% α-linolenic acid) as their sole source of fat compared to those fed corn oil or safflower-seed oil.[105] In this study, linseed oil was just as effective as fish oil in the suppression of mammary gland tumorigenesis. In carcinogen-treated rats, the feeding of a high-fat diet consisting of blackcurrant oil (16% α-linolenic acid) or one consisting primarily of fish oil (14% EPA, 8% DHA, 4% α-linolenic acid), reduced mammary gland tumorigenesis when compared to a corn oil control; the fish oil diet, however, was considerably more effective than the blackcurrant oil diet in inhibiting these tumorigenic processes.[114] Growth and metastasis of a transplantable mammary carcinoma in mice has been reported to be reduced in mice fed a linseed oil diet (50% α-linolenic acid) compared to mice fed a corn oil diet.[115] Growth of a transplantable mammary carcinoma in mice has been reported to be greater when linoleic acid was the sole source of dietary fat compared to tumor growth in mice fed only α-linolenic acid as a fat source.[90]

In contrast to these reports supporting an inhibitory effect of α-linolenic acid, compared to linoleic acid, in mammary gland tumorigenesis, long-term feeding of a diet containing linseed oil (24% α-linolenic acid) as a sole source of fat did not alter "spontaneous" mammary gland tumorigenesis in mice when compared to mice fed a corn oil diet.[87] Thus, in the few studies that have compared this short chain omega-3 polyunsaturated fatty acid (i.e., α-linolenic acid) with longer chain omega-3 polyunsaturated fatty acids (i.e., EPA and DHA), it appears that the longer chain omega-3 fatty acids are more effective in the suppression of mammary gland tumorigenesis. Clearly, additional studies are needed to evaluate this comparison more precisely and to define the role of shorter chain omega-3 polyunsaturated fatty acids in relation to that of omega-6 polyunsaturated fatty acids in this tumorigenic process.

Other types of dietary fat were also examined for their effects on mammary tumor development in rodents. Trans fatty acids (partially hydrogenated soybean oil and cotton-seed oil, 50:50, approximately 38% trans fatty acids) modified carcinogen-induced rat mammary gland tumorigenesis during the promotion stage no differently than cis fatty acids (olive oil, cocoa butter, coconut oil, 50:48:2). Both of these fat mixtures

reduced mammary gland tumorigenesis to a comparable degree when compared to a corn oil diet.[43,45] The content of trans and cis fatty acids in these diets was similar. Thus, trans fatty acids appear to behave like saturated fatty acids in modifying rat mammary gland tumorigenesis. Feeding high levels of medium-chain triglycerides from hydrolysis of coconut oil, which contains over 70% of fatty acids of 12 carbons or less in length, to carcinogen-treated rats during the promotion stage reduced mammary tumor development compared with rats fed a high level of corn oil.[41,56] There is no consistent and/or compelling evidence for an important role of cholesterol in rodent mammary gland tumorigenesis.[71,116,117]

Relatively few research groups have focused on the relationship between dietary fat and metastasis from mammary tumors in experimental animals. Some studies report that the amount and/or type of dietary fat can significantly effect mammary tumor metastasis in rodents[22-24,100,101,118,119]; others report to the contrary.[111,112,120-122] It has been reported that increasing the linoleic acid content of the diet increases the rate of mammary tumor cell metastasis (cells injected SC).[118,119] Metastasis of transplantable mouse mammary tumors (cells injected via tail vein) was reported to be increased by a diet rich in beef tallow compared to a diet containing low amounts of beef tallow; a different rate of metastasis between low- and high-fat diets of vegetable origin (corn oil) was not observed.[24] In rats bearing transplantable mammary tumors, the metastasis rate increased as the corn oil content of the diet was increased,[22,23,101] and was dependent upon the age of the animals[23] and the duration of high-fat feeding.[22] Mice fed a diet rich in trans fatty acids (soybean oil and cotton-seed oil, 50:50, hydrogenated) had fewer mammary tumor cell metastases than mice fed a diet rich in cis fatty acids (olive oil, cocoa butter, coconut oil, 58:40:2).[100] The fatty acid concentrations were similar in both diets. Metastasis of transplantable mammary tumors increased in rats fed high corn oil diets compared with animals fed high olive oil or beef tallow containing diets.[101] In other studies, comparisons of corn oil versus beef tallow,[24] safflower-seed oil versus coconut oil,[121] or corn oil versus fish oil[112,122] showed no differences in metastases of mouse[24,121] or rat[112,122] mammary tumor cells. These results provide evidence that the amount and/or type of dietary fat not only affects mammary tumor growth in rodents, but in addition, may affect mammary tumor cell metastatic processes as well.

The relationship between dietary fat and calories in mammary tumor development in experimental animals

The relationship between dietary fat and calories in mammary gland tumorigenic processes in rodents has been examined by several research groups. Clearly, caloric restriction has a profound and consistent inhibitory activity on the development of rodent mammary tumors.[8,16,34,48,50,57,60,97,123-142] The critical question is whether or not the enhancement of mammary gland tumorigenesis by hyperalimentation with fat results from the metabolic activity of the fat per se or is due to an excessive energy intake. It has been reported that a low-fat, high-calorie diet increased carcinogen-induced rat mammary gland tumorigenesis more than a high-fat, low-calorie diet.[34] Indeed, a linear direct relationship between the degree of energy restriction (10% to 40%) and the inhibition of carcinogen-induced rat mammary gland tumorigenesis (promotion stage) has been observed.[124] The diets were adjusted so that animals among the energy-restricted groups consumed comparable amounts of fat. It was concluded from these studies that dietary energy may be a greater determinant than dietary fat in enhancing mammary gland tumorigenesis.

Evidence supporting the concept that enhancement of mammary gland tumorigenesis by a high-fat diet is a function of fat per se (independent of calories) is as follows. In animals fed isocaloric low- and high-fat diets (ingesting virtually identical amounts of calculated kcal/day), the development of mammary tumors has been reported to be greater in animals fed the higher level of fat. This has been reported by many laboratories using carcinogen-induced mammary tumors in rats[13,16,18,19,41,61,77] and "spontaneous" mammary tumors in mice.[7,143,144] Furthermore, the noteworthy differences in mammary tumor development in mice and rats fed diets containing identical levels of fat but differing in fat composition (e.g., corn oil vs. beef tallow, primose oil, palm oil or fish oil, etc.) is often provided as additional direct, noncaloric activity of fat, because such diets are purportedly equicaloric. In those reports comparing isocaloric low- and high-fat diets, caloric consumption was computed using the customary Atwater values for metabolizable energy of 4, 4, and 9 kcal/g for carbohydrate, protein, and fat, respectively. It has been reported that the Atwater value of 9 kcal/g of dietary fat may not be appropriate for rodents consuming large quantities of fat and that a value of 11.1 kcal/g or approximately 124% of the expected value, is a more appropriate estimate of metabolizable fat energy in such animals (since 9 kcal/g is the maximum energy yield of fat via calorimeter analysis, the relative energy yield from protein or carbohydrate would be less than the customary values of 4 kcal/g).[145] This suggestion is not novel, for many years ago it was proposed that as the fat content of the diet increased, the amount of recovered energy is also

increased.[146] Shortly thereafter, this concept was applied to tumorigenic (skin) processes.[147] In essence, more energy may be recovered or retained within the animal from a calorie of dietary fat than from a calorie of dietary carbohydrate or protein, in animals consuming increased amounts of fat. If this concept is correct, the assessment of the available energy from low- and high-fat diets, as calculated in virtually all past studies has been erroneous. Such diets may not be isocaloric. Thus, animals consuming the higher of the "isocaloric" fat diets may be acquiring greater amounts of metabolizable energy in addition to consuming a larger quantity of fat. In support of this viewpoint, it was reported recently that when low- and high-fat "isocaloric" diets (corn oil) were fed to female rats (rats were individually housed and fed, the caloric consumption calculated by using Atwater values was virtually identical in the two groups of rats), a significant increase in body weight gain (approximately 13%) was observed in the rats fed the higher level of fat.[16] That different types of fat of similar caloric value and fed at identical levels (e.g., corn oil vs. beef tallow, fish oil, etc.) can differentially influence mammary gland tumorigenesis in rodents would appear to support a calorie-independent activity of fat. However, recent studies have provided evidence that animals consuming fats such as fish oils have reduced carcass energy accumulation compared with animals fed equal amounts of corn oil.[148-150] Thus, different types of fat fed at the same level may not yield comparable amounts of usable energy.

One of the most impressive observations emerging from the studies of calories and fat and rodent mammary gland tumorigenesis has been the profound impact of caloric restriction on this tumorigenic process. In general, the longer the duration of caloric restriction, the greater the inhibition of mammary tumor development.[125] Furthermore, inhibition of mammary gland tumorigenesis by caloric restriction can occur even when calories are restricted late in the tumorigenic process.[15,125,135] In a recent study, energy consumption restricted by 10%, 20%, and 30% during the promotion stage of rat mammary gland tumorigenesis resulted in an approximate 30%, 60%, and 90% inhibition, respectively, of this tumorigenic process.[15] In this study, the diets were adjusted so that animals in each group consumed the same amount of fat, protein, vitamins, and minerals. Using a similar experimental design, fat consumption restricted by 10%, 20%, and 30% resulted in only an approximate 7%, 18%, and 25% inhibition, respectively, of mammary gland tumorigenesis.[15] In this latter study, the diets were "isocaloric" (using Atwater values) and adjusted so that the levels of linoleic acid, protein, vitamin, and mineral consumption were similar among the different diet groups. In these studies,[15] if the relative risk (RR) of the 20% fat diet is set as 1.0, a 25% reduction in fat consumption results in a RR of 0.8. In contrast, if the RR of ad lib feeding is set as 1.0, a 25% reduction in caloric consumption results in a RR of 0.25. Thus, a small reduction in energy consumption will substantially reduce mammary gland tumorigenesis in rodents; extremely large reductions in fat consumption are required to produce a quantitatively equivalent inhibition of this tumorigenic process.

In view of the numerous reports demonstrating impressively a direct positive relationship between caloric consumption and rodent mammary gland tumorigenesis,[15,16,35,124,151] recent studies have focused on whether or not modification of energy expenditure could influence this tumorigenic process. Exercise is a potential means of achieving this goal.[57,59,152-156] In a recent study using the carcinogen-induced rat mammary tumor model (promotion stage), it was reported that voluntary exercise (free access to an activity wheel) of rats fed a high-fat diet resulted in a reduction in mammary tumor development to a degree comparable to that observed in control sedentary rats fed a low-fat diet.[57] Paradoxically, those rats that exercised the most had the smallest reduction in mammary tumor development. In contrast, in a study using virtually the same experimental animal model, involuntary exercise (motorized treadmill) of rats fed a high-fat diet resulted in an enhancement of mammary tumorigenesis[59] that was directly related to the intensity of exercise.[153] Although these reports would appear to be contradictory, an increase in mammary gland tumorigenesis was observed when the intensity of exercise or stressful activity increased. Stress, including exercise-induced stress, can readily increase secretion of pituitary prolactin,[157,158] a known hormonal stimulant of rodent mammary gland tumorigenesis.[159] Extremely severe stress can have the opposite effect.

Using a different experimental mammary tumor model, that is, the carcinogen-treated mouse model, it was reported that moderate exercise (rotating-drum treadmill) reduced the incidence of mammary tumors in mice fed a high-fat diet but had no effect on tumor incidence in mice fed a standard low-fat diet.[154] Interpretation of this study is difficult as the exercised animals consumed significantly less diet than the sedentary controls. In other studies, voluntary exercise has been reported to reduce the incidence of "spontaneous" mouse mammary tumors and to have no effect or enhancement of metastasis of a transplantable rat mammary tumor.[155] Clearly, using exercise to modify

energy expenditure in the clarification of the relationship between energy (calories), fat, and mammary gland tumorigenesis is an important and much needed experimental objective. However, such studies are difficult and can be beset by an array of known (e.g., endocrine) and no doubt many unknown confounding variables.

The restriction of caloric consumption not only suppresses the development of mammary tumors in experimental animals but, in addition, appears to block the significant differential in mammary gland tumorigenesis between rats fed low- and high-fat diets.[16,48,57,160] Thus, in carcinogen-treated rats, reducing energy consumption by 30%,[160] by 25%,[57] or by as little as 12%[16] of that consumed by ad lib fed controls negates the significant mammary tumor stimulation of a high-fat diet. Furthermore, groups of carcinogen-treated rats fed low- and high-fat diets and restricted in food consumption to a level equivalent to that consumed by the animals that consumed the least amount of food showed no differences in mammary tumor development.[48] Thus, these studies provide compelling evidence that the enhancement of mammary gland tumorigenesis in rodents by a high-fat diet is a phenomenon dependent upon an ad lib type of feeding protocol. These studies clearly do not support an often quoted study published many years ago indicating that animals consuming a high-fat diet, compared to those consuming a low-fat diet, had an increased incidence of mammary tumors regardless of the level of caloric intake.[8] Virtually all of the studies that have reported an enhancing effect of a high-fat diet on mammary gland tumorigenesis in mice and rats used an ad lib type of feeding protocol.

Dietary fat and the growth and metastasis of human breast carcinomas maintained in immune deficient mice

Studies have been designed to evaluate the influence of dietary fat on development of human breast carcinomas maintained in immune deficient mice (athymic nude mice).[161-167] All human breast carcinomas for these studies were derived from well-characterized cell lines. High-fat diets (20% corn oil) were reported to stimulate the growth of MDA-MB231 human breast carcinomas when compared with growth of tumors in mice fed a low-fat diet (5% corn oil).[165] The growth of MCF-7 and MDA-MB231 human breast carcinomas in mice was greatest in mice fed high levels of a vegetable oil (corn oil), intermediate in mice fed high levels of an animal fat (butter), and lowest in mice fed high levels of a fish oil diet (Menhaden oil).[165-167] Inhibition of growth of MCF-7 human breast carcinomas in mice fed evening primrose oil or fish oil compared with a low-fat commercial chow control also was reported.[163] Diets rich in fish oil suppress the growth of MX-1 human breast carcinomas in mice compared with diets rich in corn oil.[161,162,164]

The MDA-MB435 human breast carcinoma cell line is one of the few human breast carcinoma cell lines that metastasize (lung) when maintained in athymic nude mice. The growth and metastasis of this cell line have been reported to be increased in athymic nude mice fed a high-fat diet (23% corn oil) compared to mice fed a low-fat diet (5% corn oil).[168] After surgical excision of these carcinomas, carcinomas recurred at the excision site more rapidly in the mice fed the high-fat diet compared to those fed the low-fat diet; metastasis of these cells was positively correlated with recurrent carcinoma weight.[169] Athymic nude mice fed a high-fat diet (23%) with three different levels of linoleic acid (12%, 8%, and 2%) were inoculated with MDA-MB435 human breast carcinoma cells. Carcinoma growth rate and metastasis were significantly greater in the mice fed the 12% and 8% linoleic acid diets compared to mice fed the 2% linoleic acid diet.[170] High dietary levels (23%) of corn oil (CO) and fish oil (FO) blends (18% CO/5% FO, 11.5% CO/11.5% FO, and 5% CO/18% FO) were fed to athymic nude mice bearing MDA-MB435 human breast carcinomas. Carcinoma growth and metastasis were significantly suppressed in mice fed the highest amount of fish oil compared to mice fed the highest level of corn oil.[171] In summary, it appears that the growth and metastasis of human breast carcinomas in immune deficient mice can be significantly influenced by the amount and type of dietary fat, an effect quantitatively and qualitatively very similar to that which occurs in rodent mammary tumors.

Suppression of mammary gland tumorigenesis: Via products of lipid peroxidation?

There is ample evidence that the addition to culture media of polyunsaturated fatty acids can cause cytolysis of cultured human breast carcinoma cells.[172,173] Not all polyunsaturated fatty acids, however, have the same ability to lyse breast carcinoma cells. For example, γ-linolenic acid, arachidonic acid, and EPA with 3, 4, and 5 double bonds, respectively, were the three most effective cytolytic agents among the fatty acids tested, whereas DHA with 6 double bonds was the least effective.[172,173] Linoleic acid and α-linolenic acid, with 2 and 3 double bonds, respectively, had intermediate cytolytic activities. It was concluded that the cytotoxic

potential of these fatty acids was dependent on, but not directly proportional to, the number of double bonds in the carbon chain.[172,173] The human breast carcinoma cells examined in these studies were the ZR-75-1 cell line. Because the presence of polyunsaturated fatty acids in cell membranes increases the susceptibility of cells to lipid peroxidation, the role of secondary products of lipid peroxidation in cytolysis of these cells was examined. The results of these studies showed that the effectiveness of a given fatty acid in lysing breast carcinoma cells correlated with the intracellular thiobarbituric acid-reactive substances (TBARS) content; γ-linolenic acid and arachidonic acid generated the most TBARS and DHA produced the least TBARS.[174] Iron and copper catalase accelerated the rate of fatty acid-induced cytolysis; antioxidants such as vitamin E, vitamin A, butylated hydroxyanisole, uric acid, and lipid peroxidation inhibiting enzymes, for example, superoxide dismutase, glutathione peroxidase suppressed the rate of cytolysis.[175,176] Indomethacin, an inhibitor of endoperoxide formation, did not reduce cytolysis or TBARS concentrations.

TBARS are comprised of malondialdehyde and an array of malondialdehyde-related chemicals that are produced during lipid peroxidation. Subsequent studies provided additional evidence in support of a fatty acid-lipid peroxidation process in fatty acid-induced cytolysis of ZR-75-1 human breast carcinoma cells as there was an increase in the occurrence of conjugated diene and/or hydroperoxyl (or peroxyl) groups in the fatty acids of the fatty acid (plus iron) treated breast carcinoma cells.[177] Also, an increase in the Schiff's reaction (detects aldehydes and carbonyl compounds) in these cells, after treatment with fatty acids (plus iron), was observed.[178] The identification of the hydroxy fatty acids from the hydroperoxyl groups was attempted by GC-MS in a recent study; 15-, 12- and 8-OH 20 carbon and 13-OH 18 carbon fatty acids were observed in the fatty acid (plus iron) treated ZR-75-1 human breast carcinoma cells.[179] Thus, these results provide compelling evidence that the ability of specific fatty acids to lyse human breast carcinoma cells in vitro correlated with the extent of generation of secondary products of lipid peroxidation from the fatty acids added to the culture media.

The feeding of diets rich in polyunsaturated fatty acids to experimental animals in an effort to determine the effect of such diets on mammary gland tumorigenesis has been performed by a number of laboratories as previously described in this communication. In summary, dietary fats (oils) rich in long chain omega-3 polyunsaturated fatty acids, for example, the fish oils (high concentrations of EPA and DHA); those fats (oils) rich in γ-linolenic acid, for example, evening primrose oil; and those fats rich in α-linolenic acid, for example, perilla oil and linseed oil all have been reported to reduce mammary gland tumorigenesis in mice or rats when compared with corn oil fed controls. At issue is whether or not these fats (oils) inhibit mammary gland tumorigenesis in vivo via the generation of secondary products of lipid peroxidation as described in the in vitro studies cited earlier. To date, I am not aware of any reports describing changes in levels of secondary products of lipid peroxidation in tumors of animals fed evening primrose oil, perilla oil, or linseed oil. In contrast, there is reported evidence that the feeding of fish oil (Menhaden oil) diets to athymic nude mice bearing xenografts of MDA-MB231 human breast carcinoma cells, while suppressing carcinoma growth, also increases the level of secondary products of lipid peroxidation (TBARS) in the carcinomas when compared to corn oil fed controls.[165] Furthermore, a dose response relationship between the amount of fish oil fed, carcinoma growth suppression, and the accumulation of carcinoma TBARS was observed in these studies.[166] The inhibitory effects of the fish oil diet on human breast carcinoma growth in athymic nude mice could be reversed, or at least partially reversed, by the addition of large amounts of antioxidants to the fish oil diets; addition of the antioxidants to the diet reduced the amount of TBARS in the tumor tissue.[165,166] It was evident in these studies, that the increased accumulation of secondary products of lipid peroxidation in the tumor tissue, as a function of feeding diets high in fish oil, was due, at least in part, to increased ingestion of these products.[180] Thus, suppression of growth of human breast carcinomas maintained in athymic nude mice by dietary fish oil appears to be due, at least in part, to an increased accumulation in the tumor tissue of secondary products of lipid peroxidation.

The mechanism by which secondary products of lipid peroxidation retard or inhibit mammary tumor growth processes in vitro and/or in vivo is not certain. Secondary products of lipid peroxidation (i.e., TBARS) are capable of decreasing cell proliferation through damaging cell membranes, by changing cellular composition and/or cytoskeleton assembly. These modifications in the molecular architecture of the membrane can lead to the inactivation of membrane transport systems and/or membrane bound enzymes.[181-183] This phenomenon may adversely affect the entering of cells into the cell cycle, or it may accelerate their exit (i.e., cell death). Furthermore, secondary products of lipid peroxidation can decrease tumor cell survival by inactivating polymerase reactions,[184]

forming inter- and/or intramolecular linkages between amino acid sulfhydryl groups and biomolecules DNA, RNA, and proteins,[185] and inhibiting polyamine synthesis.[186] Such processes may not only result in inhibition of cell proliferation but may also lead to an increase in cell death. Indeed, it has been reported that dietary fish oil inhibits mammary tumor growth processes by increasing mammary tumor cell death rather than suppressing mammary tumor cell proliferation. This has been demonstrated in rodent mammary tumors and in human breast carcinomas maintained in athymic nude mice.[92,187]

Whereas secondary products of lipid peroxidation may be an important mechanism by which long chain omega-3 polyunsaturated fatty acids suppress mammary gland tumorigenesis, other mechanisms are also possible. For example, the effect of these fatty acids on eicosanoid metabolism has been examined extensively. PGE_2/PGE_1 ratios in mammary tumors from fish oil fed rats have been reported to be lower than such ratios in mammary tumors obtained from control rats fed corn oil or sunflower-seed oil diets.[104,110,188] PGE_2, TXB_2, and/or 6-keto-$PGF_{1\alpha}$ concentrations have also been reported to be lower in mammary tumors in fish oil fed rats than in tumors from control rats[104,108,110,188]; these metabolites have also been shown to be reduced in mammary tumors of rats fed a diet containing primarily EPA as its fat source compared to animals fed a diet consisting solely of linoleic acid as a fat source.[189] PGE_2, TXB_2, and 6-keto-$PGF_{1\alpha}$ are all metabolic products of the action of cyclooxygenase on arachidonic acid. It appears, therefore, that these metabolites are reduced in mammary tumors of animals fed diets high in EPA and/or DHA. PGE_2 has been reported to stimulate normal and neoplastic mammary gland cells in vitro.[190,191] Additional mechanisms by which long chain omega-3 polyunsaturated fatty acids could suppress mammary gland tumorigenesis is via inhibition of fatty acid synthesis[192,193] and a reduction in energy accumulation/utilization.[148–150]

Stimulation of mammary gland tumorigenesis: Via products of lipid peroxidation?

As discussed in the preceding section, there is evidence that lipid products derived from the activity of cyclooxygenase on arachidonic acid, for example, PGE_2, can be stimulatory to mammary gland tumorigenesis. Also discussed in the preceding section is the evidence that secondary products of lipid peroxidation can be cytolytic to mammary tumor cells. Considerably less information is available, however, as to whether or not secondary products of lipid peroxidation can stimulate mammary tumorigenic processes. Mutagenicity of autoxidized linoleic and α-linolenic acids, for instance, the oxidized methyl esters and hydroperoxide derivatives, has been reported.[194] It is conceivable, therefore, that such products of lipid peroxidation can act as chemical initiators of mammary gland tumorigenesis. Diets rich in polyunsaturated fatty acids, however, do not appear to enhance or augment the initiation stage of mammary gland tumorigenesis.[26–32] Such evidence does cast doubt for a role of secondary products of lipid peroxidation as chemical initiators of mammary gland tumorigenesis, although such observations do not rule out the possibility. Because the vast majority of research studies have demonstrated that high levels of dietary fat enhance the promotion stage of mammary gland tumorigenesis, rather than the initiation stage, it would seem prudent to determine whether or not secondary products of lipid peroxidation are capable of stimulating cell proliferation processes. Unfortunately, there are very few reports addressing this potentially important issue. The first compelling evidence for a role for secondary products of lipid peroxidation in stimulating cell proliferation was reported in 1984 when it was shown that autoxidation products of polyunsaturated fatty acids, for instance, 13-hydroperoxylinoleic acid (13-HPODE) and 13-hydroxylinoleic acid (13-HODE), stimulated colonic mucosal cell DNA synthesis and ornithine decarboxylase activities in rats when the fatty acids were administered intrarectally.[195] Linoleic acid or arachidonic acid, in contrast, did not have stimulatory activities. In subsequent studies, it was reported that the minimal requirement for colonic cell stimulation was the presence of an oxidized functionality adjacent to a carbon–carbon double bond.[196] More recently, it was reported that oxygenated products of linoleic acid could markedly enhance growth factor-induced cell proliferation. 9-Hydroxylinoleic acid (9-HODE) (or 9-HPODE) and especially 13-HODE (or 13-HPODE), when provided to Balb/c 3T3 cells in vitro, markedly augmented epidermal growth factor (EGF)-induced DNA synthesis of these cells when compared to linoleic acid.[197] Similar results were obtained with the use of Syrian hamster embryo fibroblasts.[198] In the rodent mammary gland system, it is known that linoleic acid can stimulate proliferation of epithelial cells in vitro and that this fatty acid can enhance the proliferative responses of these cells to growth factors such as EGF and insulin.[190,199] While the cyclooxygenase products PGE_2 and PGE_1 can stimulate these cells in vitro,[190,191] linoleic acid can stimulate proliferation of these cells independently of the products of the cyclooxygenase enzyme system.[190] An oxidative derivative of linoleic acid, via arachidonic

Table 1. Effect of Linoleic Acid, 9-Hydroxylinoleic Acid (9-HODE), and 13-Hydroxylinoleic Acid (13-HODE) on Proliferation of Mouse Mammary Gland Epithelial Cells Maintained in a Collagen Gel Cell Culture System

Groups[a]	Number of Wells	Mean DNA μg/well ± SE
Controls	59	3.36 ± 0.32*
Linoleic acid	60	3.32 ± 0.34‡
9-HODE	61	2.25 ± 0.29
13-HODE	59	4.69 ± 0.38†

[a] Mammary gland epithelial cells were obtained from 2-month-old female Balb/c mice. The cells were grown in a collagen gel serum-free chemically defined cell culture system. Insulin and EGF were added to the culture media at a concentration of 1.0 μg/ml and 1.0 ng/ml, respectively. Linoleic acid, 9-HODE, and 13-HODE were added to the culture media at a concentration of 2.0 μg/ml. The cells were grown for 7 days; culture media was changed daily. DNA levels/well were determined by fluorometric analysis.[190]

* vs. † $p = 0.025$.

‡ vs. † $p = 0.019$.

acid, that is, 5-, 12-, or 15-hydroxyeicosatetraenoic acid (5-, 12-, or 15-HETE), can also enhance growth factor (EGF and insulin)—induced proliferation of rodent mammary gland epithelial cells in vitro and in concert with PGE_2 can completely substitute for linoleic acid in this proliferative response.[200] In studies just completed in our laboratory, we assessed the potential of 9-HODE and 13-HODE for their ability to stimulate proliferation of primary mouse mammary gland epithelial cells in a collagen gel cell culture system. 13-HODE stimulated cell proliferation more than did the parent fatty acid, that is, linoleic acid (Table 1). In contrast, 9-HODE inhibited proliferation of these cells. Recent data from our laboratory also provide evidence that 13-HODE concentrations in the mouse mammary gland are increased in animals fed high levels of a corn oil diet,[201] a diet that has been shown to stimulate growth of the normal mouse mammary gland.[202] These results provide intriguing evidence that certain oxidative products of polyunsaturated fatty acids may be more important than their parent compounds as mitogenic stimuli to normal and neoplastic mammary gland epithelial cells. The degree to which these oxidative products are derived from enzymatic processes (e.g., lipoxygenase) or by nonenzymatic processes (i.e., from the generation of free radicals) is not known.

Whereas the limited data on the oxidative products of polyunsaturated fatty acids as potential efficacious stimulators of cell proliferation processes are certainly intriguing, there are a few reports suggesting that such products may not be important in these processes. For example, the feeding of autoxidation products of linoleic acid or secondary oxidation products of linoleic acid to rats at the time of carcinogen treatment, or commencing after carcinogen treatment, did not significantly modify the development of mammary tumors in these animals.[203] Other reports providing evidence that high-fat diets, compared to low-fat diets, do not result in increased levels of conjugated dienes or TBARS (corn oil diet, mouse mammary glands)[17] or increased 5-HETE concentrations (safflower-seed oil/coconut oil diet, mouse mammary glands)[170] certainly cast doubt on this relationship. Indeed, 15-HPETE has been reported to be cytolytic to MCF-7 human breast carcinoma cells in vitro.[204] The negative results of feeding high levels of antioxidants postcarcinogen treatment to experimental animals, in an effort to modify the promotion stage of mammary gland tumorigenesis, also raises questions regarding the importance of this relationship.[98,205–207] In contrast, others have reported suppression of mammary gland tumorigenesis by feeding high levels of antioxidants commencing after carcinogen treatment,[208–211] and still others have reported an increase in TBARS in the mammary glands of rodents fed high levels of polyunsaturated fatty acids (corn oil diet).[212] Thus, it is readily apparent from the foregoing that the role of secondary products of lipid peroxidation in the stimulation of mammary gland tumorigenesis is uncertain. The studies provided to date, however, clearly provide a sufficient impetus to examine these products in this tumorigenic process thoroughly. A schematic diagram illustrating the potential role of 5-, 12-, 15-HETE, PGE_2, and, in particular, 13-HODE as stimulatory second messengers in mammary gland tumorigenesis is shown in Figure 1.

Although there is a vast array of published reports clearly documenting that the type and amount of dietary fat can significantly modulate the development and/or growth of mammary tumors in experimental animals, it is readily apparent that few research groups have addressed directly the role of lipid peroxidation in this tumorigenic process. Unfortunately, inconsistences of data in such studies are apparent. For example, as cited earlier, some groups report that antioxidants can significantly alter experimental mammary gland tumorigenesis,[208–211] observations that would support a role for lipid peroxidation, whereas others were unable to demonstrate any effects of antioxidants in this tumorigenic process.[98,205–207] Such inconsistencies and other inconsistencies, as cited in this review, could be due to differences in animal models, for example, species and strain differences, whether the mammary tumors developed "spontaneously" or were induced and/or differences in diet composition, and so forth. That tissues from different species can differ

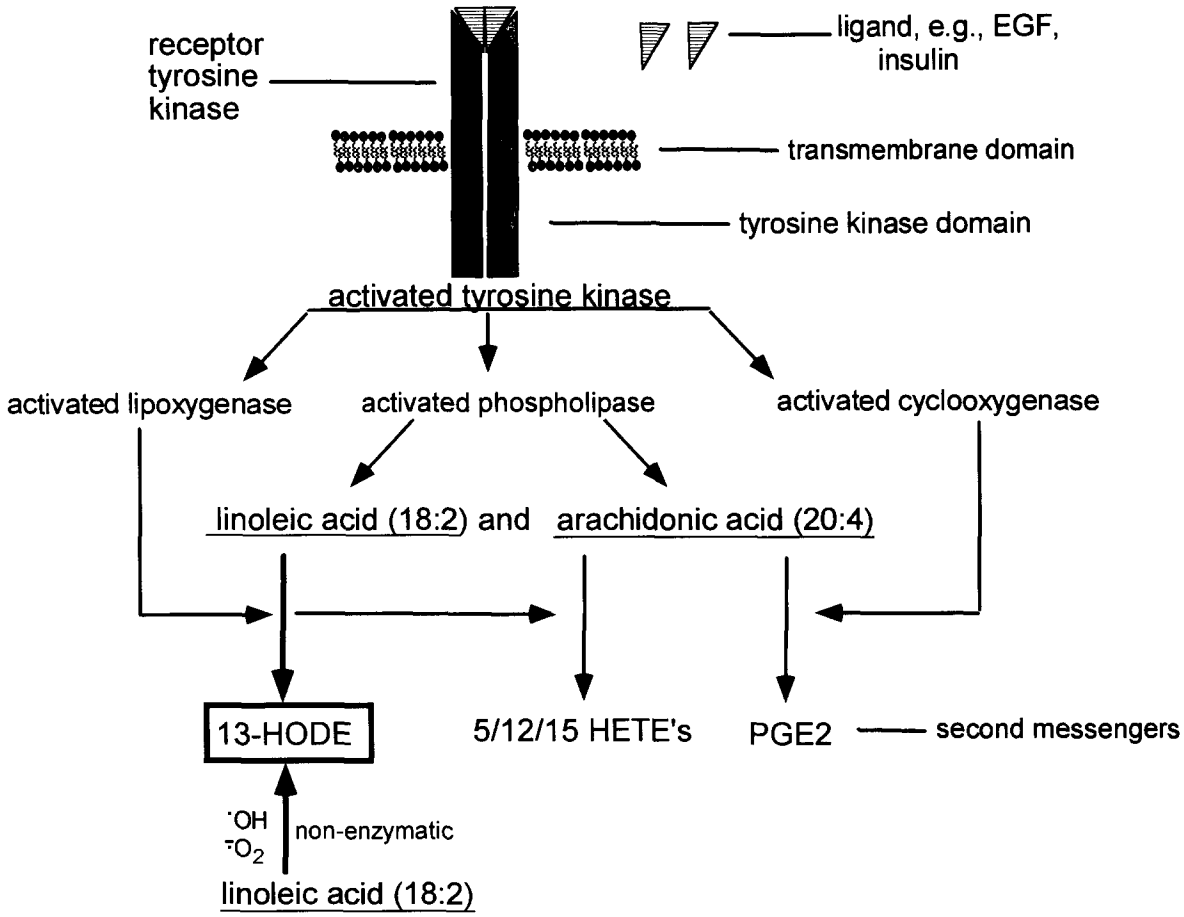

Fig. 1. 13-Hydroxylinoleic acid (13-HODE), a potentially important second messenger in the simulation of mammary gland tumorigenesis in animals fed a high-fat, high-linoleic acid diet. In this figure, a schematic outline is provided that integrates dietary fat (linoleic acid), peptide growth factors, and intracellular second messengers in the stimulation of mammary gland tumorigenesis by high-fat (linoleic acid) diets. A proposed scenario is as follows: (a) critical mitogenic mammary gland/tumor peptide growth factors, for example, EGF, insulin and so forth, bind to their specific membrane receptors and, as a result, activate receptor tyrosine kinase activities; (b) activated tyrosine kinase activities, in turn, enhance phospholipase, cyclooxygenase, and/or lipoxygenase activities; (c) activation of these enzymes facilitates the generation of important intracellular mitogenic second messengers, for example, 5/12/15 HETEs, PGE_2, and perhaps most importantly, 13-HODE. 13-HODE may be more important than 5/12/15 HETEs or PGE_2 in this scenario because the feeding of high levels of a linoleic acid-rich diet does not consistently or substantially change levels of arachidonic acid in normal or neoplastic mammary gland phospholipids or triglycerides. In contrast, such a diet consistently and substantially increases levels of linoleic acid in the phospholipid and triglyceride components of these tissues. Excess tissue levels of linoleic acid can result in excess tissue levels of 13-HODE, via nonenzymatic and/or enzymatic processes and sharply enhance the efficacy of mitogenic peptide growth factors. Thus, substantially increased tissue levels of 13-HODE, with or without increased levels of HETEs and/or PGE_2, could provide the critical mitogenic stimulus to normal and neoplastic mammary gland epithelial cells in animals fed a high-fat, high-linoleic acid-containing diet.

widely in their ability to form arachidonic acid or linoleic acid metabolites are highlighted in reports showing EGF stimulation of PGE_2 and $PGF_2\alpha$ synthesis from arachidonic acid in Balb/c 3T3 fibroblasts; lipoxygenase-derived arachidonic acid metabolites (5/12/15 HETEs) in these cells were detected only in low levels.[197] In contrast, in Syrian hamster embryo fibroblasts, EGF did not stimulate the synthesis of prostaglandins from arachidonic acid but sharply increased the synthesis of linoleic metabolites 9-HODE and 13-HODE.[198] In both cell types, however, the linoleic acid metabolites (9-HODE and 13-HODE) enhanced EGF stimulation of DNA synthesis two- to fourfold. In our studies, using primary mouse mammary gland epithelial cells, 13-HODE, but not 9-HODE, significantly enhanced the mitogenic activities of EGF and insulin (Table 1). These results, coupled with our recent report showing significantly increased 13-HODE concentrations in mammary glands from mice fed a high-fat (linoleic acid) diet,[201] suggest that 13-HODE could be

the critical chemical mediator of fat (linoleic acid)-hyperalimentation enhancement of mammary gland tumorigenesis. Thus, increasing linoleic acid levels in cells via feeding high-fat (linoleic acid) diets and perhaps even via feeding diets in excess (ad libitum feeding) could result in increased cellular levels of 13-HODE via nonenzymatic or enzymatic (lipoxygenase) processes and consequentially an enhancement of the efficacy of activities of critical mitogenic peptide growth factors (Figure 1). It is important to point out, however, that the biological activities of oxidative metabolites of linoleic acid may vary considerably from cell type to cell type. For example, 13-HODE has been reported to be antiproliferative in epidermal cells.[213] The directly opposite proliferative activities of 13-HODE on epidermal and mammary epithelial cells could explain the contrasting effects of dietary linoleic acid on epidermal and mammary gland tumorigenesis, that is, increasing dietary linoleic acid levels stimulates mouse mammary gland tumorigenesis, but inhibits mouse skin tumorigenesis.[214]

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