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EFFECT OF DIETARY FAT ON THE GROWTH OF HUMAN BREAST CARCINOMA TRANSPLANTED TO THE ATHYMIC NUDE MICE: ROLE OF LIPID PEROXIDATION IN THIS GROWTH PROCESS

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ABSTRACT

EFFECT OF DIETARY FAT ON THE GROWTH OF HUMAN BREAST CARCINOMA TRANSPLANTED TO THE ATHYMIC NUDE MICE: ROLE OF LIPID PEROXIDATION IN THIS GROWTH PROCESS

By

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In athymic nude mice, altering the levels and/or types of dietary fat, markedly influences the development and growth of transplanted human breast carcinomas (MCF-7 and MDA-MB231 cell lines). This phenomenon has also been demonstrated in an array of spontaneous, carcinogen-induced, transplantable benign and carcinomatous experimental mammary tumor systems. In general, diets high in unsaturated fatty acids (i.e., corn oil) were more effective enhancers of mammary tumorigenesis than diets high in saturated fatty acids (i.e., butter). In contrast high-fat diets with elevated amounts of long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found in certain fish oils (i.e. menhaden oil), did not enhance mammary tumorigenesis, and had a suppressive effect on tumor growth.

Various mechanisms have been proposed for this fish oil-induced inhibitory action; we have concentrated our efforts on lipid peroxidation. Our results demonstrate that this neglected mechanism, at least in part, is responsible for the suppressive action exerted by fish oils (menhaden) on the growth of human breast carcinomas (MDA-MB231) maintained in athymic nude mice. In addition, evidence is provided that in the preparation of fish oil diets, the addition of antioxidants at the amounts recommended by the American Institute of

Nutrition, or even substantially higher levels, did not completely suppress oxidative deterioration of experimental diets. In terms of tumor growth kinetics, the question arises as to what aspect of tumor growth dynamics can be affected by dietary fish oils: tumor cell proliferation or tumor cell loss. Our preliminary results suggest that tumor cell loss is more significantly affected than tumor cell proliferation in human breast carcinoma transplanted to athymic nude mice fed a diet high in fish oil compared to a diet high in corn oil. This increase in cell loss may partly explain why tumor growth is suppressed in animals fed diets high in fish oil compared to diets high in corn oil.

Dedication

To the memory of my grandparents

Juan and Luz Maria Guzman

whom in earth and now in heaven

will always guide my steps.

(Both died victims of cancer)

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I would like to give a special thank you for my grandparents, Juan and Luz M. Guzman, who are no longer here but who encouraged me to get a quality education and guided my steps toward this goal. This dissertation is dedicated to their memory.

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INTRODUCTION

Numerous authors have reported that there is a strong association between dietary fat intake and cancer prevalence (see 1 for review). Various epidemiological studies indicated that the consumption of high levels of dietary fat by humans is associated with increased incidence of cancer, particularly of the breast (2), colon (3) and prostate (4). In addition, in experimental animal studies, mammary tumorigenesis enhancement by high levels of dietary fat has been achieved in virtually every rodent model that has been examined (5-8). In these studies, the type and amount of fat ingested influences the occurrence and development of mammary tumors. High dietary levels of polyunsaturated fats have been more effective in enhancing mammary tumorigenesis than high levels of saturated fats (9-11). It has been suggested that linoleic acid may be the fatty acid primarily responsible for the tumorigenesis enhancing effect of the polyunsaturated fatty acids (12-14). It seems that in order to achieve maximal tumor growth a requirement for essential fatty acids (EFA) must be met (12). This requirement appears to be in the range of 3% to 8% of the total kilocalorie intake for rodent mammary tumor production (12). It is believed that once the EFA requirement is met, further enhancement of tumorigenesis depends on the amount and not the type of fat, although evidence exists that may challenge these criteria (15). Recent evidence has pointed to linoleic acid and arachidonic acid as

the most necessary fatty acids for mammary tumor growth in rats (14). It is also important to mention that linoleic acid is often included in synthetic tissue culture media of normal and neoplastic cell lines in order to obtain rapid cell proliferation and optimal cell growth in vitro (16).

Moreover, diets high in fat have been reported to have an immunosuppressive action, especially when high in polyunsaturated fats (17). Diets high in polyunsaturated fatty acids (PUFA) enhance immunosuppressive therapy following renal transplantation (17). Additionally, there is evidence that feeding linoleic acid prolongs skin allograft survival in rodents (18). This immunosuppressive activity of PUFAs, primarily those high in linoleic acid, has been suggested as a possible mechanism by which polyunsaturated fats enhance tumorigenesis (19,20).

Another type of polyunsaturated fat that has been studied in relation to mammary carcinogenesis is fish oil (11). Mammary tumors in rats fed diets rich in polyunsaturated long-chain fatty acids [such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], which are abundantly found in fish oils, resulted in a marked inhibition of growth (21,22). The mechanism(s) for this tumor suppressive action has not been clearly elucidated. In most of the earlier studies, menhaden oil was used as the only source of dietary fat. Menhaden oil is a very poor source of linoleic acid and the possibility of a linoleic acid deficiency in the menhaden oil-fed animals cannot be ruled out. The effect of fish oils in the generation of different series of hormone-like substances (prostaglandins) as compared to corn oil has been a popular proposed mechanism for the suppressive action exerted by fish

oils (19-22). Although seemingly important, this extensively studied prostaglandin mechanism may not be the sole one operating in this complex process of tumor growth enhancement. It has been reported that prevention of prostaglandin synthesis has not suppressed the inhibitory effects of PUFAs (23). When inhibitors of cyclooxygenase (indomethacin) or lipoxygenase (nordihydroguaiaretic acid) were added to neoplastic cells along with the fish oil, there was no decrease in the inhibitory effects of the oil. This suggests that there is no major involvement of prostaglandins or leukotrienes in mediating fish oil inhibition of tumor growth.

Another attractive mechanism that has not been adequately examined in relation to the fish oil exerted growth suppressive action on tumors is lipid peroxidation. Considering that the mammary tumor cell growth-promoting effects of fat have been closely associated with PUFAs, it is conceivable that certain PUFAs and/or their oxidative metabolites may have an important effect on mammary tumor cell proliferation (15,24).

In relation to this, it is known that antioxidants are effective suppressors of lipid peroxidation radicals and/or oxygen radicals. Thus, various laboratories have added different antioxidants to diets high in PUFAs; for example, vitamin E plus selenium (25), vitamin A derivatives (26), and butylated hydroxytoluene (BHT) (27) to decrease free radical production. Interestingly, the addition of antioxidants to diets high in PUFAs has been reported to inhibit dimethylbenzanthracene (DMBA) induced mammary gland tumorigenesis in rats (25-27). This antioxidant-induced inhibitory action on mammary tumorigenesis is contrasted with reports providing evidence of a cytostatic/cytolytic action of certain PUFAs and/or their oxidative

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products in an in vitro (16) and in an in vivo (15,28) setting. In these experiments, PUFAs underwent lipid peroxidation, generating an increased amount of secondary products of oxidation restricting cell proliferation and/or increasing cell loss resulting in a diminished tumor growth. Given the nature of the fatty acid composition of fish oils, this mechanism seems a very attractive one to explain the fish oil exerted tumor growth suppressive action compared to rodents fed diets high in corn oil.

Another important kinetic feature of tumorigenesis, in addition to tumor cell proliferation, which has not been adequately examined, is tumor cell loss. There is evidence that the increase in growth (size) of rodent mammary tumors as a result of feeding diets rich in PUFAs could not be solely accounted for by an increase in tumor cell proliferation, which suggests an important role of cell loss on tumor growth (29,30). The study of dietary fat effects on tumor growth dynamics (cell loss vs cell proliferation) has been neglected. An understanding of these kinetic phenomena might result in more effective cancer therapies.

Most of the experiments designed to examine the effect of dietary fat on mammary tumorigenesis have been carried out using rodent models, in order to evaluate the effects of dietary fats on human tumorigenesis, studies utilizing human tissue are essential.

Our experimental model consists of human breast tumor cell lines (MDA-MB231), an estrogen receptor negative cell line derived from pleural effusions of a patient with medullary carcinoma of the breast and (MCF-7), an estrogen and progesterone receptor positive cell line derived from a pleural effusion of a patient with an infiltrating

ductal breast carcinoma transplanted subcutaneously to female athymic nude mice. The nude mouse is a mutant deficient in T-lymphocytes and therefore suitable for foreign tissue grafts without rejection. This model facilitates the study of human breast cancer in an in vivo setting. It is also a means of providing important information on the influence of dietary manipulations on the in vivo growth of human breast cancer.

According to the two-stage theory of carcinogenesis (31), tumors are initiated by a mutational change in the cellular genome. This stage can occur rapidly and is essentially irreversible. Subsequent proliferation of tumor cells is a slower process (promotion), and it is thought to depend considerably on local environmental conditions. This concept offers the chance of preventing or suppressing carcinogenesis by modifying the cellular environment during the promotional stage of carcinogenesis. This can occur by creating an unfavorable or even hostile environment for proliferation and growth of tumor cells. The understanding of the role of dietary fat in human tumor cell metabolism during tumorigenesis may be a very relevant step to accomplish the suppression of malignant growth. Experiments and studies conduscent to a better understanding of nutritional influences on kinetic parameters in tumor biology are of great importance. Research involving nutrition-mediated cellular changes may provide new and different avenues of effective treatment for cancer, and may serve as a pivotal stage for new approaches against other degenerative diseases.

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REVIEW OF LITERATURE

It is very well established that the level and type of dietary fat can significantly influence the development and/or growth of mammary gland tumors in experimental animals (see 1 for review). In addition the amount and/or type of dietary fat can markedly affect the growth of human breast carcinoma (cell lines) maintained in immune deficient mice (2-7). Increasing the level of fat in the diet results in an increase in mammary gland tumorigenesis (1). Dietary fats containing large quantities of omega 6-polyunsaturated fatty acids [i.e. corn oil ≈ 50% linoleic acid (18:2n-6)] are the most effective enhancers of the tumorigenic process while fats containing high amounts of omega 3-polyunsaturated long-chain fatty acids [i.e. menhaden oil, which contain elevated concentrations of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] are less stimulatory and often suppressive to mammary gland tumorigenesis.

Since the mid-1930's, fish oils have been reported to have a suppressive effect on tumor growth (8,9). Four decades later, in the early seventies, further evidence was reported on the reduction of the incidence of spontaneously-developing mammary carcinomas in female C_3H mice fed diets high in fish oil (menhaden oil) when compared to mice fed diets high in corn oil or safflower oil (10). Since then, inhibition or suppression of mammary gland tumorigenesis has been achieved by feeding fish oil in carcinogen-induced rat mammary

carcinoma model (11-17) and in a carcinogen-induced mouse mammary carcinoma model (18). In these studies corn oil or safflower oil containing diets were used as controls. Moreover, diets high in fish oil (menhaden oil) have been reported to suppress growth of BN472 transplantable rat mammary carcinoma when compared to tumor-bearing rats fed a sunflower seed/cacao butter/olive oil diet (19). Also supplementation of laboratory stock diet with fish oil (MaxEPA) has been shown to suppress growth of R3230AC transplantable rat mammary carcinoma (20). Furthermore, the growth of the IX transplantable mouse mammary carcinoma has been reported to be reduced in mice fed high dietary levels of fish oil (menhaden oil), compared to mice fed a diet high in corn oil (21). In three of these studies (11-13), mammary gland tumorigenesis in rodents consuming high dietary levels of fish oil (menhaden oil, 20% by weight) was substantially less than that in animals consuming a diet very low in total fat (corn oil, 0.5-3.0% by weight). The fish oil diets used in these studies were rich in omega-3 long chain polyunsaturated fatty acids 20:5n-3 (EPA) and 22:6n-3 (DHA). Feeding rats a diet in which the fat content is virtually only EPA (4.7% by weight, 20:5n-3/0.3%, 18:2n-6) substantially suppressed carcinogen-induced mammary gland tumorigenesis when compared to a diet in which the sole source of fat was linoleic acid (LA, 18:2n-6, 5% by weight) (22).

Possible Mechanisms Involved in the Fish Oil-induced Tumor Suppression

The modulatory activities of omega-3 PUFAs on eicosanoid metabolism have been the most extensively studied mechanism to explain the inhibitory or suppressive activities of omega-3 PUFAs on mammary gland tumorigenesis. PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$ are metabolic products of

the action of cycloxygenase on arachidonic acid (20:4n-6). The PGE_2/PGE_1 ratios in mammary tumors from fish oil (menhaden oil or Max EPA) 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 (15,21,23). Concentrations of metabolites such as PGE_2/TXB_2 and/or 6-keto-$PGF_{1\alpha}$ have also been reported to be lower in mammary tumors of fish oil-fed rats than in tumors from control rats (15,16,21,23). These eicosanoid metabolites have also been shown to be lower 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 the source of fat (24). In addition plasma PGE_2 and TXB_2 levels have also been shown to be reduced in mammary tumor-bearing rats fed a fish oil diet compared to plasma levels in rats fed a sunflower seed oil diet (20). Therefore, these metabolites are lower in mammary tumors of rodents fed diets high in omega-3 long chain PUFAs (i.e. EPA and DHA). Despite these results, Rice-Evans and Burdon (25) reported that prevention of prostaglandin synthesis failed to suppress tumor growth inhibitory actions of fish oils. When inhibitors of cycloxygenase (indomethacin) or lipoxygenase (nordihydroguaiatetic acid) were provided along with the fish oil, no decrease in tumor growth inhibitory actions of the oil was observed.

Other mechanisms have also been examined in relation to the inhibitory effects of fish oil on tumor growth. For example, rats fed a diet high in fish oil (type not specified), compared to those fed sunflower seed, had reduced liver microsomal fatty acid desaturase activities (Δ^5 and Δ^6 desaturase). No significant decrease in the

activity of these enzymes was observed when rats were fed a diet rich in linseed oil (26). Both $\blacktriangle^5$ and $\blacktriangle^6$ desaturase activities are important in the metabolism of linoleate to arachidonte, $\blacktriangle^6$ desaturase is believed to be rate limiting for these sequential reactions. Dietary fish oil (menhaden oil) has been reported to reduce the activity of rat liver fatty acid synthase compared to a diet rich in safflower seed oil (27). Fatty acid synthase is an important lipogenic enzyme system involved in the synthesis of palmitic acid from acetyl CoA and malonyl CoA. Evidence has also been provided indicating that fish oil diets (menhaden oil), compared to corn oil diets, may decrease plasma triglycerides and free fatty acids in rats possibly by increasing the activity of skeletal muscle lipoprotein lipase (28). Lipoprotein lipase stimulates the hydrolysis of triglycerides to free fatty acids thus possibly increasing the availability of free fatty acid substrates for skeletal muscle oxidative processes. Diets high in fish oil (menhaden) have also been reported to influence hepatic phase I and phase II detoxifying enzyme systems (e.g. mixed function oxidases, glutathione-s-transferase, etc.) (29). In mammary gland chemical carcinogenesis, this modification of hepatic enzymes may be particularly relevant when fish oil diets are fed during the time period of carcinogen treatment. Modulation of hepatic enzymes can markedly influence carcinogen metabolism, thus, influencing the carcinogenesis process itself.

Another mechanism that has been largely ignored is the effect of dietary fish oil on energy accumulation. In animals fed high levels of fish oil (menhaden) a reduced carcass energy and reduced adipose tissue was observed when compared to animals fed equal amounts of corn oil or

lard (30,31). In addition, Mermier and Baker (32) reported that fatty acids, utilized for tumor energy and growth, are derived from host tissue, and it is known that fatty acid composition of tissues is influenced by dietary fat (1). In relation to this aspect of energy efficiency, Pariza and Boutwell (33) introduced the terms metabolizable energy and recovered energy. These terms differ in that energy is lost during the conversion of metabolizable energy into recovered energy. These authors also mentioned that the extent of energy loss depends on the energy source. These terms were described studying different energy sources, for instance more energy from carbohydrate is lost during this conversion than is lost when fat is used as an energy source. Nevertheless, it seems likely that certain fats such as corn oil may have a more efficient use of energy than others (i.e. menhaden oil) hence contributing more to nourishment and growth of neoplasms than do fish oils. In accordance with this hypothetical concept, it has been reported that there is a reduced lipogenic activity plus an increased mitochondrial efficiency in rats fed menhaden oil compared to rats fed corn oil (34-36). These studies provide evidence of a menhaden oil effect on intermediary metabolism. Tisdale (37) reported that EPA can induce a blockage on the production of a metabolic factor at the level of the adipocyte involved in cancer cachexia.

Fatty acids are important in the maintenance of cell membrane structure and are key determinants of membrane bound enzyme activity and receptor expression. It is recognized that an important role in the regulation of enzymatic activity and receptor expression by membranes is played by the constitutive lipids (38). In addition, the oxidation of membrane phospholipids in tissues can decrease their

fluidity, thus affecting membrane permeability, osmosis and activity of certain bound enzymes, receptors and transport systems (39-41). It has been reported that omega-3 fatty acids increase spontaneous release of cytosolic components from tumor cells (42). This probably occurs because of the unique shape of the omega-3 PUFAs (EPA, DHA), which when incorporated into cellular constituents of tumor cells, are able to change properties associated with the plasma membrane. The authors suggested that dietary manipulation may be used to enhance tumor cell permeability and contribute to tumor cell destruction. Tumors obtain a substantial amount of fatty acids from the host (43), which are primarily supplied from circulating free fatty acids (44), the mixture of fatty acids provided by the host (after only minor structural modifications) is incorporated into the complex lipids formed by the neoplastic cells (45). If the composition of circulating fatty acids can be sufficiently changed, the phospholipids formed will have a different fatty acid composition and possibly altered physical and functional properties. It is conceivable that the change in membrane fatty acids and its consequences can be sufficient to alter the tumor growth pattern or increase its sensitivity to certain forms of therapy. For example, fatty acid modification might make the neoplastic cell more sensitive to adriamycin chemotherapy. Adriamycin has been reported to accumulate in neoplastic cells enriched with polyunsaturates (46). In addition, fatty acids (LA, EPA) have been reported to modulate gene expression in human breast cancer cells (47). Fatty acids can exert these functions directly and, therefore, may themselves be important regulators of another possible mechanism: the immune response (48). The concentration and the degree of unsaturation

of dietary fats have been reported to influence several indices of immune status in a number of animal models (49-56). Diets high in certain PUFAs have demonstrated immunosuppressive potential (51). Furthermore, diets high in PUFAs enhance immunosuppressive therapy following renal transplantation (57). In addition there is evidence that feeding PUFAs high in linoleic acid prolongs skin allograft survival in rodents (58). In contrast, conflicting results regarding the effects of dietary omega-3 PUFAs in animal models were reported and only limited human studies on this topic have been conducted (49-51). Nevertheless the omega-3 enhancement of certain immunological parameters has been suggested as a possible mechanism for the inhibitory action of dietary fish oil (1).

Lipid Peroxidation and Fish Oils

Yet another possible mechanism for the suppressive action exhibited by omega-3 fatty acids on tumor growth is lipid peroxidation (59). Lipid peroxidation, or more specifically, the oxidation of polyunsaturated fatty acids refers to the oxidative deterioration of unsaturated fatty acids. It is a catalytic chain reaction probably initiated and propagated by free radicals (60), aided by molecular oxygen and bivalent cations. Susceptible substrates for this radical attack in biomembranes are PUFAs containing two or more double bonds (50,61). The groups between these double bonds are particularly susceptible to hydrogen abstraction (62). The radicals formed react rapidly with oxygen to form oxy-free radicals which convert to hydroperoxides (primary products of oxidation) that can break down to a wide variety of carbonyl compounds and to other radicals capable of further perpetuating the process (60). Most primary products of

peroxidation are unstable in vivo (63). The high concentrations of PUFA's in cell membranes make cells very susceptible to peroxidative damage (64,65) and ultimately the damage can be of such an extent that it can result in the death of cells, this accomplished by the interaction of peroxidation products with biomolecules of important and/or regulatory functions (39,66-69). Ingestion of a diet containing high levels of fish oil may increase the tendency for a tissue to undergo lipid peroxidation (70). Several laboratories have reported increased peroxidation in tissues of animals fed high levels of fish oil (71-77).

Secondary Products of Lipid Peroxidation

Of special interest are various aldehydes which are secondary products of omega-3 fatty acid oxidation that are highly cytotoxic, capable of producing a wide variety of inhibitory effects on cell proliferation (78-88). These aldehydes are mainly formed by β-cleavage reactions of alkoxy radicals arising from hydroperoxides of PUFAs based in phospholipids, glycolipids, triglycerides and cholesteryl esters (84). Also these aldehydes have been detected in many foodstuffs (i.e. microwave food containing fat) (87). No method has been developed that completely separates and identifies all the components of the complex mixture of substances that can possibly arise of the interaction of lipid-aldehyde species. These secondary products of lipid peroxidation consist of high molecular weight polymers and decomposed products. These decomposed products are mainly low molecular weight aldehydes (83) which can be easily absorbed from the diet. Examples of these aldehydes are alkanals, alkadienals, 2-alkenals and 4-hydroxyalkanal (89,90). Other examples of cytotoxic aldehydes that can be formed in

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relatively large quantities in vivo are 4-hydroxynonenal, hexanal and propanal (89). Of special interest is 4-hydroxynonenal, the major cytotoxic product of lipid peroxidation (84), which has been reported to suppress the expression of specific genes involved in the metabolic cascade that leads to cell proliferation (88). Although there are a variety of enzymes (i.e. aldehyde dehydrogenases) that evolved to metabolize aldehydes to less reactive forms (i.e. carboxylic acids), these species are still formed in vivo in enough quantities to modulate various physiological and/or biochemical functions (87). In addition to some of these mentioned, other aldehydes capable of producing a chromagen in reaction with 2-thiobarbituric acid (thiobarbituric reactive substances, TBARS) which can be measured by the 2-thiobarbituric acid assay for detection of lipid peroxidation (TBA assay) are 2-hexanal, 2,4-hexadienal and malondialdehyde (MDA). These products, especially MDA, have been reported to be cytotoxic to cell lines at physiological concentrations (90).

Thiobarbituric Acid Analysis

MDA is a secondary product of PUFA peroxidation and is most frequently measured by the TBA assay (91,92). The TBA assay is used for evaluating the degree of lipid peroxidation and is one of the most commonly used methods for detection of lipid peroxidation products (91,93). The TBA assay is a colorimetric test in which a pink chromagen is formed by the condensation of 2 molecules of TBA and 1 molecule of MDA (94). The MDA measurement is an indicator of the secondary products of peroxidation in the sample in question: the greater the number of double bonds in a PUFAs the greater the chances of forming more MDA molecules plus other secondary reactive substances.

MDA can react with amino and sulfhydryl groups (SH) of RNA, DNA and proteins (39,95) causing inter and/or intramolecular linkages that can lead to inactivation or damage of these biomolecules. Also MDA has been reported to inactivate DNA polymerases directly by blocking SH groups (69). Furthermore, it has been documented that MDA can accumulate in sufficient quantity in cells to exert various biological negative effects as the ones mentioned above (95).

During lipid peroxidation other compounds besides MDA (other aldehydes) are formed which may have a similar absorbance and which may also be capable of inhibiting cell proliferation by the same mechanisms as MDA (63,96). These products may be detected by the TBA assay. This is the main reason why secondary products of peroxidation are now referred to as TBARS instead of MDA as it was referred to in the past. In addition it should be mentioned that TBARS can also be formed from monoxidized PUFAs, carbohydrates and amino acids (97).

Lipid Peroxidation and Tumor Growth

Various factors may affect the oxidation of unsaturated lipids in complex biological tissues. Among these are pH, light, temperature, quantity of antioxidants present in the tissue, quantity and nature of metal ions present in the tissue, efficiency of oxidation inhibitory enzymes (glutathione peroxidase, catalase, superoxidase dismutase), presence of reducing agents in tissue membranes and fatty acid composition of the tissue (59).

Despite the variety of protective mechanisms [such as inhibitory enzymes and antioxidants (98)] against peroxidation, it still occurs in complex biological systems. If lipid peroxidation overwhelms the normally efficient cellular protective mechanisms, it can then cause

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genotoxicity or cytotoxicity and subsequently cell death (99). It is prudent to state that the interplay between enhancing and inhibiting factors of peroxidation is what probably determines its extent and its effect on biological systems.

It has been documented that various PUFAs are effective in killing cancer cells in vitro (69,81,100-118) but the specific mechanism of this action is yet to be determined (106). Peroxidation may be a very important factor in PUFA cytotoxicity and also in determining the rate of tumor cell proliferation (100). Peroxidation of lipids followed by decomposition of the peroxides into aldehydes or other secondary decomposition products may be a crucial step on the path leading to a reduction of the rate of cell proliferation and/or increasing the rate of cell loss of tumors. Such products have been shown to restrict cell proliferation and to cause cell death (119).

Various investigators (81,104,105,116-118) reported that the addition of iron salts to cell cultures increases the rate of cell death and TBARS number in cultured tumor cells fed high levels of PUFAs, suggesting the involvement of lipid peroxidation products in the mode of action. It is well known that transition metals can exert a major role in oxidative damage occurring in biological systems (41,120,121). In the case of lipid peroxidation, the metal involvement is dual: it can first react with molecular oxygen generating oxygen active species able to initiate lipid peroxidation by abstracting a hydrogen atom from the PUFAs; second, it can react directly with the hydroperoxide generating organic radicals that can initiate and propagate the peroxidation reaction (41). Also it is well established that iron and copper salts are prooxidant catalysts (120,121). At 37°C

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and a pH near 7.4, iron may become critical in promoting hydroperoxide decomposition to MDA. In this environment, iron serves the dual role as both initiator of lipid peroxidation and promoter of lipid peroxide breakdown (122). It is conceivable that in an environment with a lower pH (<6), as can possibly be found in tumors, iron may dissociate from ferritin and/or hemoglobin and increase aldehyde formation (123). In contrast, the addition of alpha-tocopherol reduces both cell killing and levels of TBARS in cell culture (101,105,125). Also, several investigators (126-128) reported that there is less lipid peroxidation and a higher level of antioxidants in the cancerous tissue as compared to adjacent normal tissue in breast cancer patients (113,128-130). In addition, a rather interesting observation was reported by L'Abbe' et al. (131), in which feeding a high fish oil diet induced higher lipid peroxidation in female rats compared to male rats. Meydani et al. (132) reported higher concentrations of plasma TBARS in older than in younger women supplemented with omega-3 fatty acids. These reports suggest a sex-age associated difference in incorporation or utilization of omega-3 fatty acids. In relation to antioxidants, earlier studies found antioxidants inhibited chemical carcinogenesis in a variety of tissues (see 133 for review). This antitumor activity exerted by antioxidants was probably due to an interference with initiating or promoting events in chemical carcinogenesis (134,135). Contrary to this mechanism, the addition of antioxidants to already malignant cells in the presence of PUFAs seems to protect the cells and enhance proliferation (100,105,124). Furthermore, there is some evidence of antioxidants enhancing cell proliferation (136,137).

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Evidence from cell culture studies also indicates that certain PUFAs such as eicosapentaenoic, docosahexaenoic and arachidonic acid have been effective in killing cancer cells, and this effect is correlated with intracellular TBARS levels (81,100,101,105,106); this killing of malignant cells is accelerated by iron and copper while inhibited by antioxidants (105,122). The generation of oxygen radicals and the stimulation of lipid peroxidation, seem to be necessary steps leading to cell death, in these experiments. Also, Burns and Wagner (138) reported that there is a heightened susceptibility of fish oil PUFA-enriched neoplastic cells to generate ethane during lipid peroxidation (138).

Does cell death cause lipid peroxidation or does lipid peroxidation cause cell death? This question has been addressed by Takeda et al. (118) and Benchekroun et al. (139). They reported that there was no evidence of any substantial rise in lipid peroxidation in response to cell killing by other mechanisms such as temperature, pH changes and non-peroxidation drug induced cell death. The addition of an antioxidant suppressed the formation of lipid peroxidation in neoplastic cells and the killing effect was inhibited (118). This report has been substantiated by results of our laboratory (158).

Other clues of the importance of lipid peroxidation in relation to cell proliferation can be derived from data about regenerating liver in which the content of lipid peroxidation products are low (140-142). This can suggest a possible involvement of lipid peroxidation products in the control of cell division (126,143). It is documented that embryonic tissue has a very low peroxidation content and it is also known that certain tumor cells resemble embryonic cells; also highly

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undifferentiated tumor cells have a very low degree of peroxidation which seems to be inversely related to the growth rate of tumors (113, 144-147). Moreover, rapidly growing normal tissue such as testis, bone marrow and intestinal epithelium are resistant to peroxidation and their lipid peroxidation product content is very low (120).

Conceivably, lipid peroxidation can inhibit tumor growth by several mechanisms; these include (1) interference with various phases of the cell cycle by damaging important cell compounds that would enable the cell to proceed normally through its cycle. For example, the inhibition of tubulin production by secondary products of peroxidation. Tubulin is the main functional protein involved in the formation of the mitotic spindle (148). (2) Inhibition of polyamine synthesis (148) which can directly affect the cell division process. (3) Increasing DNA synthesis time and decreasing the growth fraction of the tumor (149), thereby increasing overall cell cycle time. Secondary products of peroxidation have been shown to be diffusible (150,151), possessing the capability of traveling along the cells producing damage to susceptible sites through their pathway. Nevertheless some of these aldehydes are fairly stable (151) which permit them to escape the cell and attack susceptible targets far away from its originating initial site. In this manner these aldehydes can be considered second toxic messengers of the initial free radical event (89). However, tissue or cell injury caused by lipid peroxidation cannot be attributed to a single factor, since various radical species are formed with different cytotoxic, mutagenic, genotoxic and carcinogenic potential (87). Although lipid aldehyde levels in neoplastic cells or tissue may equate increased levels of absorption or production of aldehydes, it may also

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Lipid Composition of Tumors

There is evidence that tumor cells have an abnormal lipid composition (i.e. lower phospholipid content and low levels of PUFAs) as compared to their normal counterparts (60). Tumor cells have also a low level of enzymes of the cytochrome P450 system. These enzymes can initiate and propagate lipid peroxidation (60). In addition, higher levels of antioxidants have been reported in tumors as compared to comparable normal tissue (60). The low rate of lipid peroxidation and the low cytochrome P450 mediated activity can be associated with an increased rate of cell division (144). Apparently, secondary products of lipid peroxidation are inversely related to tumor growth. In related studies (152-154), it was reported that LA and oleic acid (18:1, n-9), in vitro, partially inhibited lipid peroxidation by chelating available free iron. The importance of this report to an in vivo setting remains to be determined. The content of nonesterified fatty acids in vivo may be insufficient to obtain this interesting effect. Two different investigators (80,155) have shown that lipid peroxidation products were too low to be detected or decreased in vivo when feeding diets high in linoleic acid content.

Interrelationship Between Lipid Peroxidation and Cancer

Cancer is a multistep, polygenic process involving various events. The induction phase is composed of two steps, initiation and promotion. At the initiation step, a change occurs in the cell genes resulting in a deregulation of the normal pathways. The cell is then endowed with the capacity to proliferate without control. In other words, the cell is transformed or "initiated". This occurs after a mutation, an

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amplification, a translocation of a cellular oncogene or a loss of a repressor gene. Chemical or physical mutagens can be involved in this step and free radicals are a common link in this process. Protection against the initiation phase can be provided by antioxidants. Generally the transformation is completed by successive mitosis during the promotion step. Any mechanism inducing the cell cycle will act as a promoter. Certain reactive free radicals are known to play an inducing part in cell activation (127) and are expected to act as cancer promoters. However, to further complicate the picture, various experiments in order to avoid the toxic effect of the free radicals onto the cell, the use of antioxidants was necessary to ensure the promotion phase (156). In tumor progression (the phase during which tumor cells become organized as a growing mass), it might be hypothesized that a senescent environment characterized by a high lipid peroxidation and a low antioxidant level may negatively influence tumor growth (127). Thus, it seems that free radicals can differently affect the process of carcinogenesis depending on the phase considered. In addition, to even further increase the complexity of this issue, it is conceivable that different species of free radicals may influence a specific phase of carcinogenesis in different ways. Some may be enhancers of cell proliferation while others can be highly toxic. If this concept is correct, then the importance is based on which species is formed in larger amounts by which dietary fat (which might be dependent on the specific composition of fatty acids).

Lipid Peroxidation and Cell Loss

A very interesting finding was reported by Gabor and Abraham (157) in which they state that the cancer-promoting effect exhibited by corn

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oil is mainly related to a decrease in tumor cell loss as compared with the higher cell loss resulting in tumor of rodents fed a diet high in fish oil. It has become increasingly evident over the past several years that tumors are not just a reflection of the rate of cell growth but also the rate of cell loss. Many reports utilizing high levels of dietary fish oils have demonstrated a decrease in tumor growth (see 1 and 159 for reviews). Dietary fish oils may decrease the rate of tumor growth by increasing lipid peroxidation products in tumor cells (6,7,59,158,159), which can decrease the growth fraction of the tumor and increase the percent of cell loss. Animals fed high levels of dietary fish oil will have a higher intake of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid that results in a higher content of these highly unsaturated fatty acids in the phospholipid fraction of their cell membrane (160). These fatty acids have 5 and 6 double bonds respectively. It has been documented that the autoxidation of fish oil in purified diets is rapid, within 24 hours (7,162-164). Furthermore, it has been suggested that the increase in tissue TBARS after feeding diets high in PUFAs are not formed in vivo but absorbed from the diet (7,160,161).

Recently it has been reported that lipid peroxidation plays an important role in tumor cell eradication by hematoporphyrin derivative sensitized phototherapy (165-166).

Secondary products of peroxidation when constantly in contact with a cancerous cell may induce profound damage, making it impossible for the cell to survive. The inhibitory or suppressive effect of fish oil may be due, at least in part, to lipid peroxidation formation of toxic compounds.

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Cell Loss and Tumor Growth

The growth rate of tumors is influenced by diverse cell cycle kinetic factors (1 for review), namely: 1) cell cycle time (time necessary for the tumor cells to reproduce); 2) growth fraction (part of the tumor in which tumor cells are actively proliferating); 3) cell loss (tumor cells that die or leave the tumor). The understanding of these kinetic factors and their interplay is of ultimate importance if a successful treatment approach against cancer is to be accomplished.

To date, cancer therapy has focused mainly on targeting cell proliferation with limited results in most tumors, while the phenomenon of cell loss has been largely ignored. However, cell loss is an important factor in determining the dynamic state of cell proliferation. Cell loss is a biological phenomenon which takes place in normal and neoplastic tissues (167). Usually an equilibrium exists in normal tissue of adults between cell proliferation and cell loss which results in a steady state growth pattern without any increase in tissue size. However, when cell proliferation exceeds cell loss, the result is an increase in size (hyperplasia). The innate ability to replace cells in a tissue is a highly successful evolutionary development (168), but perturbations in these processes can cause excessive loss or over-production of cells.

This review will focus on the importance of cell loss to solid (carcinomas, sarcomas, adenomas) tumor growth. Obviously, if cell loss (by means of cell death) can be enhanced in a solid tumor in such a way that it will overcome cell proliferation; solid tumor regression can be accomplished.

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Tumor cell loss can occur through various mechanisms such as exfoliation, metastasis, differentiation and necrosis (cell death). The relative contribution of the various modes of loss depends on the site of the tumor and its histological type. Tumors that have surfaces growing into a lumen such as in the peritoneum, pleurage, major blood vessel, etc. or growing at the surface of the body have a greater chance for cell loss by exfoliation than a tumor enclosed within an organ such as liver or kidney (169). Another mechanism of cell loss, metastasis, effectively promotes overall cancer spread. The circulating cancer cells have several effects on the host, one of which is the overcoming of immune resistance (170). However, most circulating cancer cells die in their journey throughout the body (171). But, if the metastatic cells are established, they behave like transplants that have the capacity to grow faster than the original primary tumor because of a shorter or absent lag phase or a shorter doubling time (172). Nevertheless, there is no evidence that cell loss by metastasis is a predominant mechanism affecting primary tumor growth. Differentiation of cells within tumors can provide another viable pathway for cell loss. For example, well differentiated tumors such as those of the skin and colon lose cells by keratinization or sloughing them off to the lumen of crypts (exfoliation), respectively (169).

Cell Death

Cell death is encountered in early embryogenesis since it is part of the morphogenesis of several organs (168). The programmed cell death (apoptosis) in the embryo is a vital process providing a strict spacial and temporal control. Its failure may result in congenital

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abnormalities of varying severity (168).

In tumor growth, there appears to be a reduction of the death index and/or a prolongation of the average life span of the cells (172). However, cell death in tumors appears to be the most relevant form of cell loss. Cell death in solid tumors can be promoted by numerous mechanisms, for example: insufficient blood supply with increasing tumor mass (169), immune reactions against the tumor (173), anomalies in cell division (174), and/or local effects of toxic products (59). Common pathways for cell injury that may reach a lethal outcome are associated with respiratory failure, inhibition of ATP synthesis, lysosomal release and alteration of membrane permeability (175). Various of these events may have a genetic component which may affect the chromosomes and ultimately DNA.

Cell Loss Measurement

Discrepancies between tumor cell doubling time and the volumetric doubling time of the tumor containing those cells led investigators to focus on the possibility of a negative effect of cell loss on tumor growth rate. Specifically, tumors are able to double in volume at a much slower rate than one would expect by monitoring cell cycle times of the tumor cells. The importance of cell loss in tumor growth has been stagnated by its measurement difficulty. An accurate assessment of cell loss in tumors has proven to be a very difficult task due to the following: it can only be measured in situations in which a tumor grows as a well defined mass which can be measured (176). In general, when carcinomas reach a large size this is often associated with a core of necrosis (dead center) and tumor growth is restricted to the outer rim, this would underestimate cell death substantially; especially if

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cell loss is equated to cell death. Additional sources of variability in relation to tumor growth are that tumors proliferate at very different rates in different areas of the same tumor. In spite of this limitation, there have been various attempts to measure cell loss from tumors. In order to estimate cell loss, Steel (176) developed a cell loss factor method. The cell loss factor measures the rate of loss of cells as a fraction of the rate at which cells are being added to the tumor volume by mitosis. Steel's cell loss factor expresses the loss of growth potential by the tumor on the basis of production.

A cell loss factor of 100% describes a tumor in which there is neither growth nor regression. If more cells are being generated than lost, the cell loss factor is less 100% and if more cells are lost than produced the cell loss factor is more than 100% and the tumor regresses. Tumor growth is affected by small fluctuations in elements affecting growth as well as by overall changes in growth patterns with time. Estimates of cell loss factor usually differ over time as tumors increase in size. There are a number of elements capable of influencing the cell loss factor calculations which can only, at best, be guides to the degree of cell loss. The estimations of the cell loss factor is an approximation and must be interpreted with reservation due to the impreciseness of the method. The simple measurement of tumor volume doubling time in the calculation of cell loss factor will underestimate cell loss due to the inclusion of blood, tissue fluid and necrotic material in the measurement of tumor volume. Impressions of the magnitude of cell loss has been obtained by comparing potential doubling times of tumors (expected doubling time without cell loss) determined from H^3-thymidine labelling indices (176-178). In other

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studies using human carcinomas, there was a marked disparity between the expected and observed doubling times, which suggested cell loss factors of around 60% (179) and in the region of 90% or higher (180). These results again point out the imprecision in the cell loss methodology (utilization of cell loss factor and isotopes of high reutilization potential). Also affected by local variations in tumor proliferative activity and varying distribution of cell cycle times in tumors.

Cell loss has also been studied with some success by following the loss of radioactivity from cells labelled with radioactive I^{125}-deoxyuridine (178-188). However, this procedure follows the same principle of the H^3-thymidine and also had similar problems as those associated with H^3-thymidine labelling technique. For example, still a wide dispersion of individual tumor doubling times among their group means providing an excessive variability. It has been argued that these two methods (H^3-thymidine and I^{125}-deoxyuridine) can provide information about different aspects of cell loss. Begg (183) reported that for tumors with a high cell loss rate, the H^3-thymidine method provides more meaningful data than the I^{125}-deoxyuridine technique. In contrast, Dethlefsen et al. (184) considered that the use of H^3-thymidine was well suited to the study of intratumor events, whereas the I^{125}-deoxyuridine was considered better suited to investigate the elimination of intact cells and necrotic products from a tumor population regardless of rate. Both I^{125}-deoxyuridine and H^3-thymidine are efficiently incorporated into DNA of tumor cells, but reutilization

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of I^{125}-deoxyuridine is much lower than that of H^3-thymidine (185-188). After incorporation into DNA, I^{125}-deoxyuridine remains bound within cells until they die (178,186). When a labelled cell dies, rapid deterioration and excretion of the DNA breakdown products occur limiting the reutilization by other cells to a minimum. In order to equate the loss of I^{125} activity to naturally occurring tumor cell death the following assumptions must be accepted as true: 1) the I^{125}-deoxyuridine is associated exclusively with the DNA (mainly of tumor cells); 2) I^{125}-deoxyuridine is not released from labelled cells while they are alive; 3) I^{125}-deoxyuridine does not damage or kill the labelled tumor cells; 4) I^{125}-deoxyuridine is rapidly broken down and excreted (mostly in the urine) following the death of labelled cells; and 5) chemical effects of I^{125}-deoxyuridine are minimal. Hence, this method has the advantage of not perturbing the tumor in question. Although this method, when used in vivo, possesses a high degree of "noise" (interfering variables) which makes it unfit to measure any cytocidal effect quantitatively. Nevertheless, it seems to be the most suitable way of determining cell loss occurring in solid tumors in vivo. It has been reported (189) that the volumes of carcinomas showing shrinkage 24 hours after being irradiated, did not correlate well with their cell loss factor numbers. The discrepancy is probably due to the fact that tumor regression depends not only on the radiosensitivity of the tissue but also on the rate of removal of dead cells by autolysis and phagocytosis. In order to attain a significant cell loss to incur into tumor regression, cell death needs to be complemented by an effective removal of dead cells. Tumor regression

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also depends on blood supply which reabsorbs products of autolysis and brings phagocytes into contact with dead cells. When cells in a tumor are not effectively removed, they remain to form a necrotic mass. The mass of necrotic tissue is included with the live tissue when measuring tumor volume; thereby overestimating tumor volume. This suggests that tumor volume is not a useful measurement in the determination of cell loss or cell loss factor. However, tumor volume as used in studies of tumor growth kinetics is a simple straightforward procedure which provides an insight into tumor growth despite its limitation. Because it is possible to measure tumor volume in vivo, it can be used to measure tumor growth over time which cannot be done by other means.

Solid tumors have high rates of cell loss (14) especially cell death. It remains to be determined how we can take advantage of cell loss to be used in solid tumor eradication. The manipulation of tumor cell membrane with nutritional substances (for example, omega-3 long chain polyunsaturated fatty acids) which may provide a hostile or unfavorable environment for tumor cell growth thereby promoting cell death is an attractive approach to this problem. As mentioned previously (151,190), Abraham et al. have proposed that dietary fish oil suppression of mammary tumor growth in mice is not a function of decreased cell proliferation but a function of increased cell death.

It is conceivable that a better understanding of tumor growth kinetic factors may enable us to shift the intricate balance from tumor growth stimulating factors to inhibiting ones. This action may prove to be a pivotal step in controlling the neoplastic expression and improving the response to therapy. This principle might be relevant in improving current therapeutic approaches and in developing or designing

newer and complementary therapeutic strategies against cancer. The biological modification of cell loss instead of cell proliferation might prove to be a valuable turn in the strategy used against cancer.

In conclusion, a better understanding of tumor growth dynamics (cell loss and cell proliferation) and its dietary manipulation studied in an vivo model of human tumors may provide important clues for effective cancer therapy.

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Effect

Chapter 1

Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels.

(Carcinogenesis 1991, 12:1231-1235)

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EFFECT OF DIETARY FAT ON GROWTH OF MCF-7 AND MDA-MB231 HUMAN BREAST CARCINOMAS IN ATHYMIC NUDE MICE: RELATIONSHIP BETWEEN CARCINOMA GROWTH AND LIPID PEROXIDATION PRODUCT LEVELS

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Running Title:

Dietary Fat and Human Breast Carcinoma Growth

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ABSTRACT

Human breast carcinoma cell lines MCF-7 and MDA-MB231 were transplanted s.c. to female athymic nude mice at 3-4 weeks of age. Seven to 10 days after transplantation, the mice were divided into groups and fed for 6-8 weeks one of the following semi-purified diets containing different amounts and types of fat, i.e., 5% corn oil, 20% corn oil, 20% butter, 19% beef tallow/1% corn oil and 19% fish (Menhaden) oil/1% corn oil. In additional experiments, the fish oil diets were supplemented with antioxidants (vitamin E, 8g or 2000 IU/kg diet plus tertiary butyl hydroquinone-TBHQ, 4g/kg diet) or ferric citrate (3g/kg diet). Tumor peroxidation product levels were assessed by measuring 2-thiobarbituric acid reactants (TBA assay). At the termination of the studies (6 to 8 weeks of diet feeding) mean human breast carcinoma volume (MCF-7 and MDA-MB231) was the largest in mice fed the 20% corn oil diet, intermediate in mice fed the butter or beef tallow diets and the least in mice fed the fish oil diet. The difference in mean tumor volumes among mice fed the 20% corn oil diet and those fed the fish oil diet was significant ($P < 0.01$). When comparing low (5% corn oil) and high (20% corn oil) fat diets, numerical increases in human breast carcinoma volume (MCF-7 and MDA-MB231) were consistently observed in the high fat diet groups but these differences were not always significant. Tumor lipid peroxidation product levels were determined on the MDA-MB231 tumors; tumor lipid peroxidation levels were significantly ($P < 0.01$) increased only in mice fed the fish oil diets. Supplementation of the fish oil diets with antioxidants (vitamin E + TBHQ) significantly reduced the level of tumor peroxidation products and significantly increased tumor volume

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($P < 0.05$). When tumor lipid peroxidation product levels in the fish oil plus antioxidant fed mice were reduced to the level of that observed in the tumors of the corn oil fed mice, no significant differences in tumor volumes were observed in these two groups. In contrast, supplementation of the fish oil diets with ferric citrate, significantly ($P < 0.05$) increased tumor lipid peroxidation product levels and decreased tumor volume. Thus, the type of dietary fat can clearly affect the growth of human breast carcinomas (MCF-7 and MDA-MB231) maintained in athymic nude mice. The consistent and significant inhibition of dietary fish oil (Menhaden) on growth of these carcinomas appears to be due to an increased accumulation of tumor lipid peroxidation products.

INTRODUCTION

In experimental animals, altering the level and/or type of dietary fat markedly influences the development and growth of mammary tumors. This has been demonstrated in an impressive array of spontaneous, carcinogen-induced, transplantable, benign and carcinomatous experimental rodent mammary tumor systems (1). In human populations, several epidemiological studies have suggested that diets high in fat may increase the risk of breast cancer development (2). To date, there is mainly epidemiological data to link dietary fat and human breast cancerigenesis. In an effort to resolve this relationship, we have embarked upon a series of studies to assess the influence of dietary fat on growth of human breast carcinomas maintained in athymic nude mice. In this communication, we report the influence of the amount (5% corn oil/20% corn oil) and type (corn oil/butter/beef tallow/fish oil) of dietary fat on growth of hormone responsive (MCF-7) (3) and hormone

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non-responsive (MDA-MB231) (4) human breast carcinoma cell lines in these immune deficient animals. In addition, we assessed the influence of the level of lipid peroxidation products in these carcinomas in relation to carcinoma growth. Lipid peroxidation is an oxidative deterioration of unsaturated fatty acids that give rise to a variety of oxidation products with the potential of interfering with cell replication and/or cell survival. The presence of higher concentrations of highly unsaturated fatty acids in certain dietary fats, e.g., fish oil brings into the context the possibility that oxidation products of these fatty acids may play an important role in the inhibitory or growth rate limiting action exhibited by these types of fat. In studies reported herein, we are providing evidence implicating lipid peroxidation products in the inhibitory action of diets high in fish oil on human breast carcinoma growth.

MATERIALS AND METHODS

Two hundred and fifty-eight female athymic nude mice (Harlan Sprague Dawley Inc., Madison, WI) 3-4 weeks old were used in these studies. The mice were housed under aseptic conditions (enclosed overhead laminar flow hood, sterilized cages, bedding and water) in a temperature controlled (24°C) and light controlled (14 hr/day) room. Surgical procedures for transplantation of tumors were performed under aseptic conditions in a laminar flow hood. Autoclaved laboratory mouse chow (Purina Mills Inc., St. Louis, MO) was fed ad libitum before and until 7 to 10 days after human breast carcinoma transplantation. Thereafter, mice were fed ad libitum semi-purified diets (Table 1). On a caloric basis, the diets contained the same amount of protein, minerals, vitamins and fiber. Energy calculations were based on 4

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Kcal/g of protein and carbohydrate and 9 Kcal/g of fat. All dietary ingredients were obtained from U.S. Biochemicals, Inc. (Cleveland, OH) except sucrose which was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA), fish oil (Menhaden) which was obtained from Zapata Haynie Corp. (Reedville, VA) and butter which was obtained from Michigan State University Food Stores (East Lansing, MI). The percentage of the predominant fatty acids (1% or greater, manufacturers specifications) of the dietary oils and fats are shown in Table 2. The diets are prepared in our laboratory weekly and stored at -20°C, individually packed with nitrogen prior to feeding. Mice were fed daily and non-consumed food discarded daily. Since the semi-synthetic diets were not sterilized, antibiotics (bacitracin, streptomycin and neomycin, 1g/liter) were added to the drinking water.

General Procedure

Palpable MCF-7 and MDA-MB231 human breast carcinomas (American Type Culture Collection, Rockville, MD) were surgically excised from female athymic nude mice and transplanted to 3-4 week old athymic nude mice, 3 to 4 slices (2x4 mm, 0.1 to 0.3 mm thick) of tumor tissue per mouse. Mice were anesthetized with sodium pentobarbital prior to transplantation; all surgical procedures were done under aseptic conditions in a laminar flow hood. An incision was made in the integument, the tumor slices were placed s.c. in the dorsum at distances from each other of at least 2 cm. One to 3 palpable human breast carcinomas provided the tissue slices for each experiment; care was taken to randomize the carcinoma slices from pooled tumor specimens.

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Since the MCF-7 human breast carcinoma is dependent upon estrogen for growth in athymic nude mice, mice bearing these tumors were implanted with a single estrogen pellet (2 mg 17 B-estradiol/38 mg cholesterol) s.c. and were given estrone in their drinking water (0.5 mg/L). The preparation of the hormone pellets is as previously described (5). The MDA-MB231 human breast carcinoma is hormone independent; such tumors do not require supplemented hormones for growth in athymic nude mice. Mice were placed on the experimental diets 7 to 10 days after carcinoma transplantation. This period allows the carcinoma grafts to become established in the host animals before the onset of experimental dietary treatments. Athymic nude mice bearing human breast carcinoma grafts were fed the experimental diets for a period of 6 to 8 weeks. At the termination of the studies, all tumors were excised and their volume determined ($V = 4/3\ TT\ r^3$) with a vernier caliper. Three separate studies were performed (Studies 1-3).

Study 1 The experimental design of this study is provided in Table 3. The purpose of this study was to determine whether or not the amount of dietary fat (5% corn oil/20% corn oil) or the type of dietary fat (corn oil/butter/beef tallow/fish oil) could differentially affect growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice.

Study 2 (Experimental design - see Table 4). The purpose of this study was to determine whether or not the amount and/or type of dietary fat affects the level of human breast carcinoma lipid peroxidation products. Excised tumors were assayed for lipid peroxidation products by measuring 2-thiobarbituric acid reactants using a modified TBA assay as described below.

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Study 3 (Experimental design - see Table 5). The purpose of this study was to determine whether or not the addition of antioxidants (vitamin E and TBHQ) or an oxidizing agent (ferric citrate) to the fish oil diets would decrease and increase, respectively, the level of tumor lipid peroxidation products and whether such changes in lipid peroxidation products could influence human breast carcinoma growth. Vitamin E (DL-tocopheryl acetate, U.S. Biochemical Co., Cleveland, OH, 8g or 2000 IU/kg diet) and tertiary butyl hydroquinone (TBHQ, Eastman Chemical Products, Kingsport, TN, 4g/kg diet) were added to the fish oil diets. Ferric citrate (Sigma Chemical Co., St. Louis, MO) was added to the fish oil diet at a level of 3g/kg diet. Excised breast carcinomas were assayed for lipid peroxidation products by the TBA assay. This study was performed twice, experiments #1 and 2.

Studies 1-3 Athymic nude mice that became ill and/or died prior to experiment termination were excluded from all studies (< 3% of the mice). All mice were weighed at the beginning and termination of diet feeding. Mean human breast carcinoma volume (cm^3), mean lipid peroxidation product levels (ug TBA reactants/g tissue) and mean body weight gains were statistically analyzed by one way analysis of variance and the Newman-Keuls multiple comparison test. Significance was set at $P < 0.05$.

TBA Assay

Subcutaneously growing human breast carcinomas were surgically excised, placed in a 0.9% NaCl solution, immediately frozen and stored at -20°C. Immediately before the TBA assay, the tumors were thawed, tumor samples were pooled yielding 10g of tumor tissue; the 10g tumor samples were used in the assay. An antioxidant was added (TBHQ, 0.2%

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of total fat) and samples were homogenized (4°C) in a polytron homogenizer (Ultra Turrax SDT 1810, Tekmar Co., Cincinnati, OH). The homogenates were transferred into prepared distillation flasks containing 2.5 ml of 4N HCl plus 0.5 ml silicone antifoam with sufficient distilled water to yield a total volume of 100 ml/flask. The distillate was collected (50 ml); 5 ml of this distillate plus 5 ml of freshly prepared 2-thiobarbituric acid solution (TBA, 0.2 N aqueous solution) were used for the assay. Samples were prepared in triplicate, heated in boiling water for 35 minutes to develop the pigment, cooled and absorbance read on a spectrophotometer (Spectronic 2000, Bausch & Lomb, Rochester, NY). The absorbance values obtained were multiplied by an adjustment factor of 6.2 (6). Results were reported as ug of TBA reactive substances per g of sample.

RESULTS

Effect of the type and amount of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in female athymic nude mice.

Mean human breast carcinoma volume of MCF-7 or MDA-MB231 carcinoma at termination of these studies was the largest in mice fed the 20% corn oil diet, intermediate in mice fed the butter or beef tallow diets and the least in mice fed the fish oil diet (Tables 3 & 4). The differences in final tumor volumes of mice fed the corn oil and fish oil diets were consistent and significant ($P < 0.01$). In mice fed the butter or beef tallow diets, final tumor volume was consistently intermediate to those fed the corn oil or fish oil diets. This decrease in tumor volume in mice fed the butter or beef tallow diets compared to corn oil, however, did not reach the 5% level of statistical probability.

When comparing a high fat diet (20% corn oil) to a low fat diet (5% corn oil), numerical increases in MCF-7 and MDA-MB231 carcinoma volumes at termination of the experiments were consistently seen in mice fed the higher level of fat (Tables 3 & 4). Such volume increases were significant ($P < 0.01$) in 1 of the 2 studies utilizing MDA-MB231 cells (Table 3). Thus, a trend toward increased human breast carcinoma volume is observed, albeit marginally significant, when these animals are fed the higher level of fat.

Effect of the type and amount of dietary fat on growth and lipid peroxidation product levels of MDA-MB231 human breast carcinomas in female athymic nude mice.

As in the preceding study, human breast carcinoma volume at termination of this study was largest in mice fed the 20% corn oil diet, intermediate in mice fed the butter diet, and the least in mice fed the fish oil diet (Table 4). The difference in tumor volumes of mice fed the 20% corn oil and fish oil diets was significant ($P < 0.01$). Numerical increases in tumor volumes were observed in mice fed the 20% corn oil diet compared to mice fed the 5% corn oil diet, however, these differences did not reach the 5% level of statistical probability.

Mean lipid peroxidation product levels (ug TBA reactants/g tissue) in the human breast carcinomas derived from the fish oil fed mice were significantly higher when compared to tumor lipid peroxidation products in each of the other diet groups ($P < 0.01$). No significant difference in the level of mean lipid peroxidation products in carcinomas derived from animals fed the 5% corn oil, 20% corn oil or butter diets was observed (Table 4).

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Influence of antioxidants and ferric citrate diet supplementation on growth of MDA-MB231 human breast carcinomas in female athymic nude mice fed a fish oil diet: correlation with human breast carcinoma lipid peroxidation products.

Experiment #1 (Table 5)

As in the preceding studies, human breast carcinoma volume at the termination of study was significantly ($P < 0.05$) reduced in mice fed the fish oil diet compared to mice fed the corn oil. The addition of antioxidants (vitamin E and TBHQ) to the fish oil diet caused a striking increase (6 fold, $P < 0.05$) in final tumor volume, a final tumor volume, however, that was still quantitatively less than that observed in the corn oil fed mice ($P < 0.05$). This increase in tumor volume in mice fed the fish oil diet supplemented with antioxidants was accompanied by a significant ($P < 0.05$) reduction in tumor lipid peroxidation products (ug TBA reactants/g tissue). The level of tumor lipid peroxidation products in the antioxidant supplemented fish oil diet fed mice was still significantly ($P < 0.05$) more than the level of lipid peroxidation products in the tumors of the corn oil fed mice. The addition of ferric citrate to the fish oil diet, in contrast, resulted in a further reduced final tumor volume ($P < 0.05$). Such a reduction in tumor volume was accompanied by a significant ($P < 0.05$) increase in the level of tumor lipid peroxidation products.

Experiment #2 (Table 5)

The study described above was repeated, the results are identical to those observed in experiment #1 with but one important exception. The addition of the antioxidants (vitamin E and TBHQ) to the fish oil

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diet caused an increase in final human breast carcinoma volume that was not significantly different than the tumor volumes observed in the corn oil fed mice. Furthermore, the level of tumor lipid peroxidation products in the tumors derived from the mice fed the antioxidant supplemented fish oil diet was not significantly different than the level of tumor lipid peroxidation products in mice fed the corn oil diet.

Effect of Diets on Body Weight Gains.

There were no significant differences in mean body weight gains among any of the dietary groups (data not shown). The only exception to this are the mice of study 3 (Table 5) who were fed the ferric citrate supplemented fish oil diet. While these mice gained weight throughout the study, their rate of gain was significantly ($P < 0.05$) less than the corn oil, fish oil or the fish oil plus antioxidant fed mice.

DISCUSSION

The results of our study demonstrate very clearly that the growth of human breast carcinoma cell lines MCF-7 and MDA-MB231, when transplanted to female athymic nude mice, can be markedly influenced by the type of dietary fat. Tumor growth is greatest in mice fed high levels of a vegetable oil (corn oil), intermediate in mice fed animal fats (butter, beef tallow) and the least in mice fed fish oils (Menhaden oil). Thus, the growth of these human breast carcinoma cell lines, as a function of the type of dietary fat, mimics what has been observed in numerous rodent mammary tumor models, i.e., diets rich in certain vegetable oils (e.g., corn oil, sunflower seed oil, safflower-seed oil) are the most stimulating to mammary tumor growth, diets high

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in fish oils (e.g., Menhaden oil) the least stimulatory and diets high in saturated animal fats are of intermediate potency in the growth stimulation of these tumors (1,7). That the rodent mammary tumor cells and the human breast carcinoma cells have similar growth responsiveness to alterations in dietary fat provides additional support for the important utility of rodent mammary tumor systems as models for the human disease.

Our observation that high levels of dietary fish oil suppress the growth of human breast carcinoma cell lines in athymic nude mice is an interesting and potentially important observation. Very recently, while our studies were in progress, other laboratories (8-12) have reported similar observations utilizing human breast carcinoma cell lines (MDA-330, MX-1 and MCF-7) in athymic nude mice. Clearly, high levels of dietary fish oil suppress mammary tumorigenic growth processes, not only in rodent mammary tumor models (13-15) but in xenografts of human breast carcinomas maintained in immune deficient laboratory mice as well. How dietary fish oil suppresses mammary (breast) tumorigenic growth processes is not known and is currently the focus of attention of a number of laboratories. Many laboratories have proposed that the inhibition of mammary tumorigenesis by dietary fish oil is via alterations in eicosanoid metabolism (13-15). Our laboratory, in contrast and as reported in this communication, has explored another potential mechanism, i.e., that high levels of dietary fish oil suppress mammary tumorigenic processes by causing an increased accumulation of cytotoxic and/or cytostatic lipid peroxidation products in the tumor tissue. In athymic nude mice fed the fish oil diets, we observed a substantial increase in the level of lipid peroxidation

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products (TBA reactants) in the breast carcinomas; a level of peroxidation products approximately 4 times greater than that observed in the breast carcinomas of mice of the other dietary groups. The addition of high levels of antioxidants to the fish oil diet (vitamin E and TBHQ) substantially reduced the level of tumor lipid peroxidation products and simultaneously caused a substantial increase in tumor growth. Indeed, when tumor lipid peroxidation products were reduced in the fish oil fed mice by dietary antioxidant supplementation, to the level observed in the tumors of the corn oil fed mice, no significant difference in tumor growth in these two groups of mice was observed. Furthermore, the addition to the fish oil diet of a pro-oxidant cation, i.e., ferric citrate, caused an increase in tumor lipid peroxidation product levels and a simultaneous further decrease in tumor growth. Thus, these data provide compelling evidence that the tumor growth suppressing activities of a diet high in fish oil is due, at least in part, to the generation of fish oil derived products of lipid peroxidation.

Of the dietary groups examined in these studies, only the fish oil fed mice had substantial increases in tumor peroxidation product levels. Animals fed low or high levels of corn oil or high levels of butter had approximately 1/4 the level of tumor lipid peroxidation products than that observed in the tumors of mice fed fish oil. The large amounts of eicosapentaenoic (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6) in the fish oil diets, compared to the corn oil or butter diet groups, is most likely the major reason for this increase in tumor lipid peroxidation products. It is well established that there is a direct relationship between the number of double bonds in a fatty acid

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and the potential formation of lipid peroxidation products; such oxidation can occur via a chemical or an enzymatic reaction (16).

How the accumulation of lipid peroxidation products in tumor tissue influences tumor growth processes is not entirely certain. The secondary products of lipid peroxidation measured by the TBA assay are reportedly capable of affecting cell proliferation. For example, these substances can inactivate polymerase reactions by blocking SH group (17). They can cause inter- and/or intramolecular linkages between amino acid sulfhydryl groups of RNA, DNA and proteins leading to the inactivation or damage of these molecules (18). They can inhibit polyamine synthesis (19). Such biochemical interactions of lipid peroxidation products with cellular macromolecules can not only reduce cell proliferation processes but can directly lead to cell death. Indeed, Abraham and colleagues have proposed that dietary fish oil suppression of mammary tumor growth in mice is not a function of decreased cell proliferation but a function of increased cell death (15). In a series of interesting studies, Horrobin and colleagues (20,21) have reported that the addition of relatively large amounts of certain polyunsaturated fatty acids, e.g., EPA, arachidonate, linolineate, to cultures of malignant cells causes a sharp increase in the generation of TBA reactive products and a parallel increase in cancer cell death. The addition to the culture media of inhibitors or stimulators of lipid peroxidation, e.g., vitamin E or iron, caused a significant inhibition or enhancement, respectively, of cell killing. Interestingly and of potential pragmatic importance is their observation that the enhancement of cell death by supplementation of polyunsaturated fatty acids is repeatedly seen in an array of cancer

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cell lines but is notably absent in normal cells (21). We are currently in the process of determining whether or not our previously reported observation of dietary fish oil induced inhibition of normal mammary gland growth processes (22) is a function of increasing the level of mammary gland lipid peroxidation products. The vulnerability of cancer cells to certain polyunsaturated fatty acids, while normal cells appear resistant as reported by Horrobin and colleagues (20,21), is an observation that merits further examination.

In summary, we report in this communication that the type of fat can influence growth of human breast carcinomas maintained in athymic nude mice. Most impressive was the consistent inhibitory effect of dietary fish oil on growth of these carcinomas. The inhibitory effect of the fish oil diets on growth of these carcinomas appears to be due to an accumulation of lipid peroxidation products in the tumor tissues.

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Table 1

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Table 1. Diet composition

Composition*	Low fat (5%) diet***	High fat (20%) diet***
Fat	5.00%	20.00%
Casein	17.10	20.17
DL-methionine	0.30	0.35
Dextrin	45.50	32.18
Sucrose	22.70	16.09
AIN Salt Mixture**	3.50	4.13
AIN Vitamins**	1.00	1.18
Cellulose	5.00	5.90
	100.00	100.00

*The calculated energy value (metabolizable energy) for the low fat diet is 3.87 kcal/g, for the high fat diets, 4.55 kcal/g.

**Alpha tocopherol level content of the diet is 0.2 g/100 g diet. Iron content of the diet is 0.004 g/100 g diet.

***The high fat diets consisted of 20% corn oil, 20% butter, 19% beef tallow/1% corn oil or 19% fish (Menhaden) oil/1% corn oil; the low fat diet consisted of only 5% corn oil.

Table 2. Predominant fatty acids in oils and fats (percentage)

Fatty acids*	Corn oil	Butter	Beef tallow	Menhaden oil
Caprylic (8:0)	--	--	--	--
Capric (10:0)	--	--	--	--
Lauric (12:0)	--	2.5	--	--
Myristic (14:0)	--	10.5	3.4	8.0
Palmitic (16:0)	10.1	31.0	25.5	28.9
Palmitoleic (16:1)	--	2.5	2.8	7.9
Stearic (18:0)	1.6	13.6	24.9	4.0
Oleic (18:1)	31.4	29.9	35.7	13.4
Linoleic (18:2)	56.3	1.8	1.6	1.1
Linolenic (18:3)	--	--	--	1.0
Eicosapentaenoic (20:5)	--	--	--	10.2
Docosahexaenoic (22:6)	--	--	--	12.8

*Fatty acid concentrations less than 1% are not included.

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Table 3. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas transplanted to athymic nude mice.

MCF-7 human breast carcinoma

Treatment*	No. of mice	No. of carcinomas	Mean carcinoma volume (cm^3 ± S.E.)
5% Corn oil	9	36	3.40 ± 0.28[a]
20% Corn oil	8	28	4.02 ± 0.58[a]
20% Butter	9	35	2.36 ± 0.32[a]
19% Beef tallow +1% Corn oil	9	35	2.36 ± 0.36[a]
19% Menhaden oil +1% Corn oil	9	36	0.20 ± 0.16[b]

MDA-MB231 human breast carcinoma

Treatment*	No. of mice	No. of carcinomas	Mean carcinoma volume (cm^3 ± S.E.)
5% Corn oil	8	24	0.52 ± 0.12[b]
20% Corn oil	8	22	1.54 ± 0.37[a]
20% Butter	8	24	1.16 ± 0.19[d]
19% Beef tallow +1% Corn oil	6	19	0.84 ± 0.23
19% Menhaden oil +1% Corn oil	7	24	0.56 ± 0.50[b]

*Female athymic nude mice were fed diets for 8 weeks, from 5 to 13 weeks of age, commencing 7-10 days after human breast carcinoma transplantation.

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Table 4. Effect of dietary fat on MDA-MB231 human breast carcinoma growth and carcinoma lipid peroxidation product levels.

Treatment*	No. of mice	No. of carcinomas	Mean carcinoma volume (cm^3 ± S.E.)	Mean carcinoma lipid peroxidation products (ug/ TBA reactants/g tissue ± S.E.)
5% Corn oil	5	19	0.20 ± 0.06	0.16 ± 0.04[a]
20% Corn oil	8	20	0.37 ± 0.11[a]	0.22 ± 0.04[a]
20% Butter	6	19	0.10 ± 0.03	0.20 ± 0.07[a]
19% Menhaden oil +1% Corn oil	7	21	0.03 ± 0.01[b]	0.82 ± 0.01[b]

*Female athymic nude mice were fed diets for 6 weeks, from 5 to 11 weeks of age, commencing 7 to 10 days after human breast carcinoma transplantation.

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Table 5. Effect of dietary fish oil plus antioxidants or iron supplementation on MDA-MB231 human breast carcinoma growth and carcinoma lipid peroxidation product levels.

Treatment*	No. of mice	No. of carcinomas	Mean carcinoma volume (cm^3±S.E)**	Mean carcinoma lipid peroxidation products (ug/TBA reactants/g tissue±S.E.)**
Experiment #1				
20% Corn oil	15	40	2.82±0.41[a]	0.44±0.01[a]
19% Menhaden oil +1% Corn oil	16	50	0.24±0.04[b]	1.41±0.07[b]
19% Menhaden oil +1% Corn oil and antioxidants (Vit E & TBHQ)	15	39	1.45±0.14[c]	0.70±0.03[c]
19% Menhaden oil +1% Corn oil + Fe citrate	16	40	0.18±0.03[b]	2.13±0.09[d]
Experiment #2				
20% Corn oil	22	55	3.26±0.82[a]	0.39±0.03[a]
19% Menhaden oil +1% Corn oil	24	60	0.34±0.04[b]	1.30±0.03[b]
19% Menhaden oil +1% Corn oil and antioxidants (Vit E & TBHQ)	25	70	2.39±0.35[a]	0.45±0.02[a]
19% Menhaden oil +1% Corn oil + Fe citrate	18	57	0.15±0.04[c]	1.52±0.02[b]

*Female athymic nude mice were fed diets for 6 weeks, from 5 to 11 weeks of age, commencing 7 to 10 days after human breast carcinoma transplantation.

**Means with different letters are significantly different at $P < 0.05$.

Chapter 2

Lipid peroxidation products are elevated in fish oil diets even in the presence of added antioxidants

(J. Nutr. 1992, 122:2190-2195)

LIPID PEROXIDATION PRODUCTS ARE ELEVATED IN FISH OIL DIETS EVEN IN THE PRESENCE OF ADDED ANTIOXIDANTS[1]

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ABSTRACT

Purified corn and fish oil diets with different types and concentrations of antioxidants were evaluated for oxidation products. In addition, a determination of different organ and carcass oxidation product levels was performed. PV[2] and TBA[2] assays were done on the diets immediately after mixing (0 h) and 24, 48 and 72 h after being fed to mice. The AIN recommended level of antioxidant addition (BHT[2], 0.02% w/w of oil) and even the addition of 100 times this level (BHT, 2.0% w/w of oil), although decreasing the level of oxidation products, failed to totally prevent oxidative deterioration in diets high in fish oil. Furthermore, other antioxidants added in excess to the fish oil diets also failed to completely suppress oxidative deterioration of the diet and, in addition, when fed daily to mice for a period of 4 weeks, caused an increased accumulation of lipid peroxidation products in certain organs (e.g., heart, skeletal muscle, mammary glands) and in the carcass. Thus, these results provide evidence that in the preparation of fish oil diets, the addition of the AIN recommended level of antioxidants, or levels even substantially higher than that recommended does not completely suppress oxidative deterioration of experimental diets.

Indexing keywords: fish oil, lipid peroxidation, antioxidants, Balb/c mice.

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INTRODUCTION

Polyunsaturated fats can quickly autoxidize at ambient or subambient temperatures (Chan 1987). In the food industry, this problem has been reduced by the addition of antioxidants and vacuum packaging of foods containing these fats (Buford-Coulter 1988). In recognition of this problem, the AIN has recommended the addition of a synthetic antioxidant at a level of 0.02%, (by weight of oil) to experimental animal diets containing polyunsaturated fats (AIN 1980). However, diets containing fish oil are more susceptible to autoxidation than diets containing other polyunsaturated fats (e.g., corn oil), because of their high concentration of long chain polyunsaturated fatty acids, e.g., EPA[2] (20:5, n-3) and DHA[2] (22:6, n-3). Fritsche and Johnston (1988) reported an extremely rapid autoxidation of diets with added fish oil as measured by PV. This report by Fritsche and Johnston (1988) plus an earlier publication by Rasheed et al., (1963), which also reported a high autoxidation in diets containing fish oil, are two of only a few publications dealing with the important issue of autoxidation in fish oil containing diets.

It is our opinion that in most studies dealing with the role of dietary fish oil in physiological or pathological processes, the process of dietary autoxidation has been largely ignored. In a recent report, our laboratory has provided evidence that the inhibitory effect of a fish oil diet on tumorigenic processes may be mediated primarily by a lipid peroxidation process (Gonzalez et al. 1991). Such evidence has provided the basis for our belief that the importance of autoxidation or lipid peroxidation has been underestimated by those researchers interested in the health impact of fish oil diets. With

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this in mind, we felt it necessary to further evaluate the issue of autoxidation of fish oil containing diets and the accumulation of oxidation products in organs of mice given a diet rich in fish oil under normal feeding circumstances.

MATERIALS AND METHODS

Diets

Purified diets (Table 1) were formulated according to a slight modification of the guidelines developed by AIN (1977). Dry components of the diet (Table 1) were mixed for 15 min and stored at -20°C until used for diet preparation. The fish (Menhaden) oil was used upon arrival with the remaining oil stored in small dark containers sealed with nitrogen gas. Mean PV of the Menhaden oil when received was 7.25, after 7 days it was 7.76 (-20°C) and after 14 days it was 10.45 (-20°C). The Menhaden oil diet was prepared the following way: 800 g of previously mixed dry ingredients were combined with 190 g of Menhaden oil and 10 g of corn oil (19% fish oil/1% corn oil). Corn oil was added to fish oil to increase the amount of linoleic acid, an essential fatty acid for normal growth and development. The use of corn oil, a polyunsaturated vegetable fat ($\simeq$60% linoleic acid, 18:2, n-6) as a control comparison for fish oil was based on its frequent use in experimental animal dietary studies. These diets, utilizing 20% fat w/w (200 g/kg diet), are considered high fat diets.

The antioxidants TBHQ[2] (Eastman Chemical Products, Kingsport, TN) or BHT (Sigma Chemical Co., St. Louis, MO) were immediately added to the fish oil at a level of 0.02% (BHT) (w/w oil) or 2.0% BHT or 2.0% TBHQ $\pm$ 8 g/kg dl-alpha tocopherol acetate (U.S. Biochemical, Cleveland,

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OH). Sufficient diet for one day's feeding was packed into small plastic zipper sealed bags. Each bag containing diet was flushed with nitrogen gas and stored at -20°C for no longer than 7 days (no significant increase in diet peroxidation is observed during the 7 day storage period).

Analysis for Thiobarbituric Acid and Peroxide Value Assays of Diet

Diets were fed (free access) to mice to expose diets to actual experimental conditions. One hundred twenty mature female mice (Balb/c), from our breeding colony, were housed in 24 cages (5 mice/cage). Mice were housed in a temperature controlled (24°C) and light controlled (14 hr. light/day) room. Our protocol complied with the Guide for the Care and Use of Laboratory Animals (NRC 1985). Triplicate samples of each diet were obtained for analysis immediately after mixing (0 h) and 6 diet samples of each diet group were analyzed for each time period, i.e. 24, 48 and 72 h after being fed.

Lipid peroxidation was determined by two methods, the PV (mmol of peroxides/kg oil) by the method of the American Oil Chemist's Society (1973) and by measuring TBARS[2] (μg TBARS/g sample) using a modified TBA assay as previously described (Gonzalez et al. 1991). Modification of the TBA assay is as follows: to 10 g of diet, TBHQ (0.2% of total fat, 2 g/kg sample) was added to diet samples before homogenization at 4°C to prevent further oxidation during the assay. The homogenates were treated with 4N HCl. A distillate was collected and combined with equal amounts of freshly prepared TBA solution. Samples were heated in boiling water, cooled and absorbance read on a Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY). Once diet samples

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were obtained, they were analyzed immediately for PV and TBARS which were done in duplicate and triplicate, respectively.

Organ and whole body analysis

Lipid peroxidation was assessed in liver, heart, kidney, mammary glands and skeletal muscle (15 animals/group) and in whole body carcass (10 animals/group) of female mice by a modified TBA assay (Gonzalez et al. 1991). Mice were fed one of the following diets daily (non-consumed diet discarded daily) for 4 weeks: 20% corn oil, 19% fish oil/1% corn oil or 19% fish oil/1% corn oil with added antioxidants, i.e., TBHQ, 2.0% w/w of oil and dl-alpha tocopherol acetate, 8 g/kg or 2000 IU/kg diet. At termination, organs were collected and stored in a NaCl solution (9g/L) at -20°C. These were analyzed for TBARS the following day. Animals used for whole body carcass TBARS were terminated by carbon dioxide inhalation and stored intact in a plastic zipper bag at -20°C and analyzed for TBARS the following day.

Statistical analysis

Results were statistically analyzed by one-way analysis of variance and the Newman-Keuls multiple comparison test (Wilkinson 1989). Significance was set at $p < 0.01$.

RESULTS

At 0 h, the PV of the corn oil diet was lower than any of the fish oil diets except for the fish oil diets containing 2.0% TBHQ $\pm$ vitamin E (Table 2). At 0 h, the PV of the non-supplemented fish oil diet was higher than the PV of the corn oil diet or the PV of each of the fish oil diets supplemented with antioxidants. This trend was observed throughout the other time periods (24, 48, 72 h). Increasing the level of BHT from 0.02% to 2.0% reduced the PV at all time points. TBHQ (2%)

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was slightly more effective than BHT (2%) in reducing PV. The addition of vitamin E to the TBHQ group further reduced, albeit slightly, PV values. PV at 0 h (immediately after diet preparation) was the lowest compared to all other time points (24, 48, 72 h) both in the corn and in the fish oil diets. Oxidation occurring between diet preparation (0 h) and after being fed for a day (24 h) was the period in which the rate of PV increase was at its highest. PV levels increased in all diet groups over time throughout the entire experimental period (0, 24, 48 and 72 h).

At 0 hour, the dietary TBARS were lowest in the corn oil diet and highest in the fish oil diet not supplemented with antioxidants (Table 3). The addition of antioxidants (BHT or TBHQ) to the fish oil diet decreased the levels of TBARS when compared to the non-supplemented fish oil diet. Increasing the level of BHT from 0.02% to 2.0% reduced TBARS at all time points. TBHQ (2%) was slightly more effective than BHT (2%) in reducing TBARS. The addition of vitamin E to TBHQ further reduced, albeit slightly, TBARS. However, at 0 and 24 h, the levels of TBARS were higher at all levels of antioxidant supplementation than that observed in the corn oil diet. Dietary TBARS values at 72 h were lower than those at 48 h except for the fish oil diet which was not supplemented with antioxidants. In all of the foregoing, the TBARS were measured at the standard wavelength of 532 nm. However, an increase in dietary TBARS was observed at wavelengths 455 nm and 495 nm at 72 h as compared to 455 nm and 495 nm wavelength readings at 0, 24 and 48 h time points (data not shown).

TBARS values of organs (liver, heart, kidney, mammary gland, skeletal muscle) and whole bodies of mice fed a fish oil diet without

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antioxidants were consistently higher than that observed in organs and whole bodies of corn oil fed mice (Table 4). The addition of high levels of antioxidants to the fish oil diet reduced TBARS values in all organs and in whole bodies (carcass). However, TBARS values in heart, skeletal muscle, mammary glands and whole bodies (carcass) of mice fed fish oil containing antioxidants were still higher than TBARS values of mice fed a corn oil diet.

DISCUSSION

The PV of fish oil diets constantly increased with time exposure to air and under normal feeding conditions. Peroxide formation is likely to occur as susceptible polyunsaturated fatty acids are available in the oil (Esterbauer et al. 1986). PV increases are catalyzed by free radical attacks at multiple sites. This might explain the observed higher rate of dietary peroxide formation attained during the 0 to 24 h period in which more susceptible sites for free radical attack are likely to be present. The addition of the synthetic antioxidant BHT (0.02% w/w oil) to the fish oil diet significantly reduced lipid peroxidation when compared to the control fish oil diet. The higher concentration of BHT (2.0% w/w of oil) was more effective than the 0.02% level. TBHQ, at the 2.0% level was slightly more effective in reducing peroxide formation. TBHQ has been reported previously to be the most effective antioxidant for fish oil (Ke et al. 1977). Nevertheless, peroxide formation still occurred in the fish oil diet even when 100 times the AIN recommended level (0.02% w/w of oil) for a synthetic antioxidant (2.0% TBHQ or BHT) was added. Since peroxides manifest a transitory nature, over time conversion to secondary products of oxidation is favored.

Secondary products of lipid peroxidation (TBARS) measured by the TBA assay also displayed an increasing trend over the first 48 h. However, this trend was not apparent at 72 h where a decline in TBARS values was observed. In contrast to PV, TBARS formation depends on specific site oxidation accompanied by serial decomposition reactions (Dahle et al. 1962). Further examination of TBARS at shorter wavelengths (455 nm and 495 nm) suggest the possibility of a subsequent conversion of these secondary products of lipid peroxidation to other related compounds, most probably aldehydes such as alkanals and alk-2-enals (Kosugi et al. 1987) in the 72 h sample. Such TBARS determined at shorter wavelengths, should be expected to increase as secondary reactions proliferate with continuing oxidation. It is also possible that the observed reductions in TBARS values at 72 h may be due, at least in part, to possible reactions between aldehydes (i.e., malondialdehyde and/or alkanals) and proteins (Cuppett et al. 1989). The addition of synthetic antioxidants to the fish oil diets resulted in a substantial decrease in dietary TBARS, as was observed for PV, i.e., 2.0% TBHQ was most effective, followed by 2.0% BHT, then 0.02% BHT. Once again, and importantly, the addition of synthetic antioxidants at a level 100 times greater than the AIN recommended level (0.02% w/w of oil) did not totally prevent the formation of oxidation products in the fish oil diet. TBARS values in the antioxidant supplemented fish oil diets were still higher than that observed for corn oil. In regard to the oxidative susceptibility of the dietary oils used in this study, it is clear that corn oil ($\simeq$ 60% linoleic acid, 18:2,n-6) is considerably less prone to autoxidation than is fish oil. This difference is no doubt due to the high content

of long chain polyunsaturated fatty acids, i.e., EPA and DHA, in fish oil; such fatty acids are absent in corn oil (Table 2).

Significant differences in lipid peroxidation were found among fish oil diets prepared from oils originating from separate batches from the same supplier (data not shown). The mean PV in freshly obtained fish oil in our studies was 7.25 (for comparison purposes, fresh stripped corn oil had a mean PV value of 0.97). This particular problem may prove to be very difficult to solve and may be due to an array of variables such as storage, processing, air and light exposure, temperature and seasonal variations (e.g. type of phytoplankton available for fish consumption). The rapid addition of a synthetic antioxidant to the oil as soon as the oil container is opened should deter or decrease further deterioration of the oil.

Organs and whole body carcasses of mice fed the corn oil and fish oil diets (± antioxidants) were also evaluated for TBARS. TBARS values obtained from certain organs and carcasses were directly related to the oxidation content of the ingested diet, although it is conceivable that this process can be further enhanced *in situ*. Even large doses of antioxidants (100x the AIN recommended level of a synthetic antioxidant and approximately 30x the amount of tocopherol acetate of that supplied by the vitamin mix) added to the fish oil diet failed to prevent the accumulation of secondary products of oxidation in certain organs (heart, skeletal muscle, mammary gland) and in whole bodies (carcass) of mice fed the supplemented fish oil diet. Lebovitz et al. (1990) reported similar results in tissue slices obtained from rats consuming a fish oil diet. They reported a decrease in secondary oxidation products in tissue slices of organs of rats receiving high levels of

dietary vitamin E, but also were unable to fully suppress the formation of these oxidation products. In contrast to what was observed in our study with heart, skeletal muscle and mammary glands, the livers and kidneys of mice fed fish oils containing antioxidants had significantly lower TBARS values than did the livers and kidneys of mice fed corn oil. Although the exact explanation is not certain, it is conceivable that at least for the liver, the capacity for storage of antioxidant lipid soluble components and/or the function of this organ in the production of lipoproteins may explain this phenomenon.

In summary, based on the results presented in this communication, the AIN recommended level of addition of a synthetic antioxidant (0.02% w/w of oil) to diets containing high levels of polyunsaturated fatty acids appears to be insufficient to completely inhibit oxidative deterioration when such diets have fish oil as their fat base. Thus, a distinct recommendation for fish oil diets compared to other polyunsaturated fats seems appropriate. Meanwhile, in order to minimize oxidative deterioration in experimental diets containing high levels of fish oil, a synthetic antioxidant should be added in quantities substantially above the AIN recommendations. PV and TBARS values should be monitored throughout all feeding experiments involving dietary fish oil. Animals must be provided a fresh diet at least every 24 h in clean food jars; the remaining non-consumed diet should be discarded. In addition, the mixing of diets in an inert atmosphere (e.g. N_2 or CO_2) should be mandatory. Freshly prepared diets should be stored in individual zipper bags in an amount sufficient for a single day's feeding and kept at -20°C until fed. These recommendations will substantially reduce, albeit not completely eliminate, excessive

oxidative deterioration of experimental diets incorporating high levels of fish oil. The multitude of research laboratories studying the effects of dietary fish oil on physiological and/or pathological processes should be cognizant of these concerns.

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Table 1. Diet Composition

Ingredients[1]	g/100 g diet[2]
Fat[3]	20.00
Casein[4]	20.17
dl-Methionine	0.35
Dextrose	32.18
Sucrose	16.09
AIN Mineral mix[5]	4.13
AIN Vitamin mix5[5]	1.18
Cellulose[6]	5.90

[1]All ingredients (dry components) were obtained from U.S. Biochemical Co., Cleveland, OH except sucrose (ICN Biochemicals, Inc., Costa Mesa, CA).

[2]High fat diets (20% w/w) consisted of 20% corn oil or 19% fish oil (Menhaden)/1% corn oil. Menhaden oil contains 50 IU/kg of dl-alpha tocopherol.

[3]Corn oil (tocopherol stripped) was obtained from U.S. Biochemical Co., Cleveland OH. Fish oil (Menhaden) was obtained from Zapata Haynie Corp., Reedville, VA.

[4]Vitamin free, high nitrogen (14.5%).

[5]J. Nutr. 107: 1340-1348, 1977.

[6]Celufil, non nutritive bulk.

Table 2. Effect of supplemented dietary antioxidants on peroxide values of fish (Menhaden) oil diets.

	Peroxide Value (mmol/kg oil)[1] Time (hours)			
Diet	0[2]	24	48	72
20% corn oil	1.33±0.17[a]	8.67±0.34[a]	11.67±0.88[a]	19.67±1.46[a]
19% fish oil/1% corn oil	13.57±1.25	45.33±2.28[d]	71.20±2.89[f]	262.67±7.27f[f]
19% fish oil/1% corn oil + 0.02% BHT[3]	6.67±0.88[c]	25.67±0.88[c]	39.67±1.46[e]	78.20±2.09[e]
19% fish oil/1% corn oil + 2% BHT[3]	4.01±0.58[b]	17.08±0.58[b]	30.67±2.48[d]	48.66±4.42[d]
19% fish oil/1% corn oil + 2% TBHQ[4]	2.07±0.29[a]	14.08±1.15[b]	22.01±0.58[c]	38.67±1.20[c]
19% fish oil/1% corn oil + 2% TBHQ[4] + 2000 IU Vitamin E[5]	1.50±0.29[a]	11.67±0.88[a]	17.05±1.16[b]	30.15±0.50[b]

Footnotes for Table 2

[1]Statistical comparisons made at each time point between diets (values are means ± SEM, n=6) determined by ANOVA and Neuman-Keuls multiple comparison test.

[2]Value with different superscript letters in the same column are significantly different at $p < 0.01$.

[3]Butylated hydroxytoluene

[4]Tertiary butyl-hydroxyquinone

[5]dl-alpha-tocopherol acetate

Table 3. Effect of supplemented dietary antioxidants on TBARS levels of fish (Menhaden) oil diets.

Diet	Thiobarbituric acid reactive substances (μg TBARS/ g diet)[1] Time (hours) 0[2]	24	48	72
20% corn oil	0.35±0.01[a]	0.49±0.03[a]	0.74±0.04[a]	0.50±0.03[b]
19% fish oil/1% corn oil	2.93±0.33[f]	6.53±0.15[f]	18.95±0.45[e]	19.06±0.67[e]
19% fish oil/1% corn oil + 0.02% BHT[3]	1.18±0.03[e]	1.68±0.14[e]	1.95±0.09[d]	0.97±0.05[d]
19% fish oil/1% corn oil + 2% BHT[3]	0.70±0.03[d]	0.95±0.04[d]	1.10±0.04[c]	0.80±0.05[c]
19% fish oil/1% corn oil + 2% TBHQ[4]	0.63±0.03[c]	0.84±0.05[c]	0.91±0.03[b]	0.47±0.04[b]
19% fish oil/1% corn oil + 2% TBHQ[4] + 2000 IU Vitamin E[5]	0.49±0.03[b]	0.67±0.04[b]	0.79±0.05[a]	0.37±0.03[a]

Footnotes for Table 3

[1]Statistical comparisons made at each time point between diets (values are means ± SEM, n=6) determine by ANOVA and Neuman-Keuls multiple comparison test.

[2]Value with different superscript letters in the same column are significantly different at $p < 0.01$.

[3]Butylated hydroxytoluene

[4]Tertiary butyl-hydroxyquinone

[5]dl-alpha-tocopherol acetate

Table 4. Effect of supplemented dietary antioxidants on TBARS levels organs in mice fed fish (Menhaden) oil diets for 4 weeks.

	Thiobarbituric acid reactive substances (μg TBARS/g tissue)[1]					
Diet	Liver[2]	Heart	Kidney	Skeletal muscle	Mammary glands	Carcass
20% corn oil	0.62±0.03[a]	0.23±0.02[a]	0.63±0.03[a]	0.30±0.03[a]	0.38±0.03[a]	1.53±0.06[a]
19% fish oil/ 1% corn oil	0.98±0.05[c]	0.55±0.03[c]	0.97±0.04[c]	0.73±0.04[c]	2.07±0.07[c]	2.95±0.08[c]
19% fish oil/ 1% corn oil + 2% TBHQ[3] + 2000 I.U. Vitamin E[4]	0.50±0.03[b]	0.35±0.02[b]	0.56±0.03[b]	0.56±0.03[b]	0.77±0.03[b]	2.01±0.05[b]

[1]Statistical comparisons of organs and carcass between diet groups (values are means ± SEM, n=10 except for heart, kidney and mammary glands for which n=6) were determined by ANOVA and Neuman-Keuls multiple comparison test.

[2]Values with different superscript letters in the same column are significantly different at $p<0.01$.

[3]Tertiary butyl-hydroxyquinone.

[4]dl-alpha-tocopherol acetate.

Footnotes

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[2]Abbreviations used: BHT, butylated hydroxytoluene; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic acid; PV, Peroxide value; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroxyquinone.

Chapter 3

Dietary fish oil inhibits human breast carcinoma growth: A function of increased lipid peroxidation

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Dietary Fish Oil Inhibits Human Breast Carcinoma Growth: A Function of Increased Lipid Peroxidation

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Running title: DIETARY FISH OIL AND HUMAN BREAST CARCINOMA GROWTH

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Abbreviations:

CO, corn oil; EPA, eicosapentaenoic acid; FO, fish oil; DHA, docosahexaenoic acid; TBA, thiobarbituric acid assay; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butyl hydroquinone

ABSTRACT

Female athymic nude mice were implanted s.c. with human breast carcinoma MDA-MB231. Seven to 10 days later, the mice were divided into groups and fed a purified diet containing the following types of fat (% of diet): 1) 20% corn oil (CO); 2) 15% CO/5% fish (menhaden) oil (FO); 3) 10% CO/10% FO; 4) 5% CO/15% FO; 5) 1% CO/19% FO and 6) 1% CO/19% FO plus antioxidants [alpha tocopherol acetate, 2000 IU/kg diet and tertiary butyl-hydroquinone (TBHQ), 2% of total fat]. The linoleic acid levels (% of diet) of the groups were 12.0%, 9.1%, 6.2%, 3.3%, 0.9% and 0.9%, respectively. After 6-8 weeks, the carcinomas were assessed for tumor volume (cm^3) and assayed for thiobarbituric acid reactive substances (TBARS). Human breast carcinoma growth was suppressed in mice consuming FO diets without antioxidants compared to mice fed CO; the greater the amount of dietary FO fed, the greater the carcinoma growth suppression ($p<0.05$). The addition of antioxidants to the FO diet significantly ($p<0.05$) reversed the FO induced carcinoma growth suppression. Concentrations of TBARS in the human breast carcinomas were increased in all the FO (without antioxidants) fed mice, compared to mice fed CO; the level of increase in TBARS was directly related to the increase in the level of FO fed ($p<0.05$). The addition of antioxidants to the FO diet significantly ($p<0.05$) reduced the concentration of TBARS in the breast carcinomas. Thus, these results provide evidence that dietary FO can significantly suppress growth of human breast carcinoma MDA-MB231, even in the presence of substantial amounts of linoleic acid (3.3% to 9.1%). The inhibitory effect of FO on growth of these carcinomas was associated with an increased concentration of TBARS in the tumor tissue. In conclusion,

dietary FO induced suppression of human breast carcinoma growth is a function, at least in part, of an accumulation of lipid peroxidation products in the tumor tissues.

INTRODUCTION

Fish oil (FO), which contains elevated levels of long chain omega-3 polyunsaturated fatty acids, in particular, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), has been shown to suppress mammary gland tumorigenesis in rodent models (1-4). In addition, FO has been shown to inhibit the growth of human breast carcinomas maintained in immune deficient animals (5-9). To explain how FO inhibits mammary tumorigenic processes, many investigators (2-4) have focused on EPA and/or DHA-induced alterations in eicosanoid metabolism. Other investigators (10-11) suggest that dietary FO inhibits mammary tumorigenesis because it may not provide a sufficient amount of essential fatty acids such as linoleic acid (18:2). Indeed, a direct relationship between the amount of dietary linoleic acid and mammary tumor growth has been clearly shown (12). In contrast, we have explored yet another potential mechanism, i.e., that high levels of dietary FO inhibit mammary tumorigenesis by causing an increased accumulation of cytostatic and/or cytotoxic lipid peroxidation products in the tumor tissue (9). In this communication, we further examine the relationship between dietary FO and growth of human breast carcinomas in athymic nude mice by pursuing the following objectives: 1) to determine whether or not high levels of dietary FO, in the presence of substantial amounts of linoleic acid, can inhibit growth of these tumors and 2) whether or not there is a correlation between tumor growth and the quantity of lipid peroxidation products in the tumor

tissues of mice fed FO.

MATERIALS AND METHODS

Animals and diet. One hundred sixty-four female athymic nude mice (Harlan Sprague Dawley Inc. Madison, WI) 4 to 5 weeks old were used in these studies. The mice were housed under aseptic conditions (enclosed overhead laminar flow hood, sterilized cages, bedding and water) in a temperature (24°C) and light (14 h/day) controlled room. All experimental procedures pertaining to the athymic mice were performed under aseptic conditions. Autoclaved laboratory mouse chow (Purina Mills Inc., St. Louis, MO) was fed ad libitum before and up to 7-10 days after human breast carcinoma transplantation. Thereafter, mice were fed purified diets (Table 1) ad libitum. The percentages of the predominant fatty acids (1% or greater, manufacturer's specifications) of the dietary oils are shown in Table 2. The diets were prepared weekly in our laboratory, individually packed in small plastic zipper-sealed bags of sufficient size for one day's feed, flushed with nitrogen, sealed and stored at -20°C. Mice were fed daily and non-consumed food discarded daily. Since the purified diets were not sterilized, antibiotics (bacitracin combined with streptomycin or neomycin, 1 g/L) were added to the distilled drinking water.

Human breast carcinoma transplantation. Palpable MDA-MB231 human breast carcinomas (American Type Culture Collection, Rockville, MD) were surgically excised from female athymic mice, cut into slices (2x4 mm, 0.1-0.3mm thick) and implanted into recipient mice. Mice were anesthetized with sodium pentobarbital (60 μg/g, i.p.) prior to transplantation. An incision was made in the integument, the tumor slices were placed s.c. in the dorsum at distances from each other of

at least 2 cm, 3 to 4 slices/mouse. Between 1 and 3 palpable human breast carcinomas were pooled to provide the tissue slices. Mice were placed on the experimental diets 7-10 days after carcinoma transplantation. This period allows the carcinoma grafts to become established in the host animals before the onset of experimental dietary treatments. Athymic mice bearing human breast carcinoma grafts were fed the experimental diets for a period of 6-8 weeks. At the termination of the studies, all tumors were excised and their volume determined ($V = 4/3\ \pi\ r^3$) with a vernier caliper. Four separate studies (1-4) were carried out, each study was a complete individual experiment.

Study 1. The experimental design of this study is provided in Table 3. The purpose of this study was to determine if varying levels of FO mixed with varying levels of linoleic acid-rich corn oil (CO) are capable of modifying the human breast carcinoma growth suppressive effect of a FO diet. In addition, we sought to determine if a relationship exists between human breast carcinoma growth and the level of lipid peroxidation products in the tumor tissue. The level of lipid peroxidation products of excised 10 g tumor composites in each group were determined by measuring 2-thiobarbituric acid reactive substances (TBARS) using a modified macro-thiobarbituric acid (TBA) assay (9).

Macro-TBA assay. Subcutaneously growing human breast carcinomas were surgically excised, placed individually in a 0.9% NaCl solution, immediately frozen (-20°C) and stored for one day. Immediately before the TBA assay, the tumors were thawed and tumor samples pooled to obtain 10 g composites. Once sufficient tumor homogenate was obtained from pooling (10 g composite), this constituted one sample. To obtain

other samples, other tumors within the same group were also pooled to form 10 g samples. Three to 5 samples (10 g composites) were done in triplicate for each group. An antioxidant [tertiary butyl-hydroquinone (TBHQ), Eastman Chemical Products, Kingsport, TN], at 0.02% of total fat, as determined by a dry column extraction method (13) was added to the samples; such samples were homogenized (4°C) in a polytron homogenizer (Ultra Turrax SDT 1810, Tekmar Co., Cincinnati, OH). The homogenates were transferred into distillation flasks containing 2.5 mL 4N HCl plus 0.5 mL silicone antifoam (Thomas Scientific Co., Swedesboro, NJ) with sufficient distilled water to yield a total volume of 100 mL/flask and distilled to collect 50 mL. Five mL of this distillate plus 5 mL of freshly prepared TBA (0.2N aqueous solution, Sigma Chemical Co., St. Louis, MO) were used for the assay. Samples were prepared in triplicate, heated in a boiling water bath for 35 min to develop the pigment, cooled and absorbance read on a spectrophotometer (Spectronic 2000, Bausch & Lomb, Rochester, NY) at 532 nm. The absorbance values obtained were multiplied by an adjustment factor of 6.2 (14). Results were reported as μg of TBARS per g of sample.

Study 2. The experimental design of this study is provided in Table 4. The purpose and experimental design of this study was similar to Study 1 but two additional dietary groups (1% CO/19% FO and 1% CO/19% FO plus antioxidants) were added. A modified micro-TBA assay (15) was used in this study in order to individually assay each excised tumor for TBARS.

Micro-TBA assay. Subcutaneously growing human breast carcinomas were surgically excised, frozen (-20°C) for one day, thawed and

individually homogenized (4°C) in the presence of an antioxidant (TBHQ, 0.02% of total fat) in a polytron homogenizer. One mL of homogenate was combined with 2.0 mL of TBA (0.375% wt/vol), trichloroacetic acid (15.0% wt/vol), and HCl (0.25N) and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the precipitate was removed by centrifugation (1,000 xg for 10 min) and the supernatant kept for analysis. The absorbance of the sample was determined at 535 nm against a blank that contains all reagents minus the homogenate. This TBA assay is a slightly modified version of the Buege and Aust assay (15). TBARS concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (16) and reported as nmoles TBARS per mg of protein. The protein concentration of tumors was determined according to the method of Lowry et al. (17).

Study 3. The experimental design of this study is shown in the legend of Figure 1. The purpose of this study was to determine if the concentration of TBARS in individual human breast carcinomas correlated with tumor size from a single FO dietary group (1% CO/19% FO). Excised tumors were individually assayed for TBARS by the modified micro TBA assay.

Study 4. The experimental design of this study is provided in Table 5. The purpose of this study was to determine if the length of time the FO diet is allowed to remain in the food jar, under feeding conditions, i.e., 24, 48 and 72 h after feeding, influences human breast carcinoma growth and carcinoma concentration of TBARS. The jars with diet were changed every 24, 48 or 72 h. Excised tumors were individually assayed for TBARS by the modified micro-TBA assay.

Statistical analyses. Data in Studies 1,2, and 4 were analyzed by one-way analysis of variance and the Newman-Keuls multiple comparison test (18). Significance was set at $p<0.05$. Data for Study 3 were examined by Pearson's correlation coefficient (18).

RESULTS

Human breast carcinoma growth. In Study 1, mean human breast carcinoma volumes (cm^3) obtained from the 4 dietary groups at experiment termination ranked (from largest to smallest) as follows: 20% CO > 15% CO/5% FO > 10% CO/10% FO > 5% CO/15% FO (Table 3). Mean carcinoma volumes in mice fed each of the 3 FO diets were significantly ($p<0.05$) smaller than the mean carcinoma volume of mice fed CO as their sole source of fat (20% CO). Body weight gains were not significantly different among the mice in the different dietary groups.

In Study 2, mean human breast carcinoma volumes (cm^3) obtained from the 6 dietary groups at termination of the experiment ranked (largest to smallest) as follows: 20% CO > 15% CO/5% FO > 1% CO/19% FO plus antioxidants >10% CO/10% FO > 5% CO/15% FO > 1% CO/19% FO (Table 4). Mean carcinoma volume in mice fed 20% CO was numerically higher than those of the 15% CO/5% FO and 1% CO/19% FO plus antioxidants fed mice, but this difference did not reach a level of significance. However, each of these 3 groups had mean carcinoma volumes which were significantly ($p<0.05$) larger than those produced by the 10% CO/10% FO, 5% CO/15% FO, 1% CO/19% FO dietary groups. Body weight gains were not significantly different among the mice in the different dietary groups.

In Study 4, mean human breast carcinoma volumes (cm^3) obtained from the 3 dietary groups at termination of the experiment ranked (largest

to smallest) as follows: 24 h - 1% CO/19% FO > 48 h - 1% CO/19% FO > 72 h - 1% CO/19% FO (Table 5). Mean carcinoma volumes in mice in which the diet (1% CO/19% FO) was changed every 24 h was significantly larger ($p<0.05$) than in the 48 h or 72 h diet groups. The mean carcinoma volume of the 48 h diet group was significantly larger ($p<0.05$) than that of the 72 h diet group. Body weight gains were significantly lower ($p<0.05$) for the 72 h diet group compared to the 24 hour diet group.

Human breast carcinoma lipid peroxidation product levels (TBARS). The ranking of mean human breast carcinoma TBARS concentrations in the 4 dietary groups of Study 1 at termination was: 5% CO/15% FO > 10% CO/10% FO > 15 % CO/5% FO > 20% CO (Table 3). Mean human breast carcinoma TBARS concentrations in mice fed each of the 3 FO diets were significantly ($p<0.05$) greater than the mean carcinoma TBARS value in mice fed CO as their sole source of fat (20% CO). In Study 2, the ranking of the mean human breast carcinoma TBARS concentrations in the 6 dietary groups at termination was: 1% CO/19% FO > 5% CO/15% FO > 10% CO/10%FO > 15% CO/5% FO > 20% CO > 1% CO/19% FO plus antioxidants (Table 4). Mean tumor TBARS concentrations were virtually identical in the 1% CO/19% FO plus antioxidants and 20% CO fed animals. Mean tumor TBARS concentration in mice fed each of the four non-antioxidant supplemented FO diets were significantly ($p<0.05$) greater than the mean tumor TBARS concentrations in mice fed the antioxidant supplemented FO and in the group fed only CO as their sources of fat. In Study 3, an inverse relationship is shown between volumes of individual human breast carcinomas and carcinoma TBARS concentrations in mice fed the 1% CO/19% FO diet ($p<0.05$) (Figure 1), i.e., the smaller the tumor

volumes, the greater the concentration of TBARS. In Study 4, mean carcinoma TBARS concentration in mice in which the diet (1% CO/19% FO) was changed every 24 h was significantly lower ($p<0.05$) than in the 48 or 72 h diet groups (Table 5). The mean carcinoma TBARS of the 48 h diet group was significantly less ($p<0.05$) than that of the 72 h diet group.

DISCUSSION

This study provides evidence that supplementation of a high fat (CO) diet with even a modest amount of FO (e.g., 15% CO/5% FO) can result in a suppression of growth of a human breast carcinoma (MDA-MB231) maintained in athymic nude mice. Other laboratories have provided evidence that FO containing diets inhibit mammary gland tumorigenesis in rodents (1-4) and suppress growth of human breast carcinomas in athymic nude mice (5-9). Such studies often utilize diets containing FO as their only source of fat. FO is extremely low in the essential fatty acid linoleic acid (18:2). It is conceivable, therefore, that the suppression of mammary tumorigenesis exerted by diets high in FO may be via essential fatty acid (linoleic acid) insufficiency. Indeed, supplementation of FO diets with modest amounts of linoleic acid, in certain mammary tumor animal models, has been reported to block (4) or partially block (19) the tumorigenic inhibitory activities of a diet high in FO. In this study, we were still able to attain growth inhibition of MDA-MB231 human breast carcinomas in athymic nude mice by feeding FO diets containing substantial amounts of linoleic acid (3.3% -9.1%). While the estimated nutrient requirement for adequate growth and reproduction of mice for linoleic acid is 0.3% wt/wt of diet (20), the level required for

optimal tumorigenic processes may be substantially higher. For example, Ip *et al*. (12) have reported that approximately 4.4% of linoleic acid in the diet is required for optimal (maximal) mammary tumor development in a rodent model. In our study, diets containing FO and substantial amounts of linoleic acid (up to 9.1% of diet) still suppressed growth of this human breast carcinoma. Thus, it is likely that dietary FO-induced suppression of growth of human breast carcinoma MDA-MB231 is not due to insufficient dietary linoleic acid.

Inhibition of growth of human breast carcinoma MDA-MB231 by dietary FO may be *via* the accumulation within the tumor of cytostatic and/or cytolytic lipid peroxidation products. Evidence presented in our study which supports this conclusion are as follows. 1) As the FO content of the diet is increased, a concurrent significant increase in carcinoma TBARS concentrations is observed. 2) Increasing the FO content of the diet decreases carcinoma growth. 3) Those carcinomas that have the highest concentrations of TBARS have the least tumor growth. 4) Allowing the FO diet to remain in the food jar for as long as 72 h, a feeding protocol that increases substantially the level of TBARS and the peroxide values in the diet (21), results in an increased accumulation of TBARS in the carcinomas and a concordant suppression of carcinoma growth. 5) The addition of efficacious antioxidants to the FO diet substantially decreases carcinoma TBARS concentrations and concurrently increases carcinoma growth. Collectively, these results, clearly support the concept that lipid peroxidation products are relevant to the process of dietary FO-induced inhibition of breast carcinoma growth. Recent evidence from our laboratory (21) indicates that dietary FO derived lipid peroxidation products are formed

extensively in the food jar under normal feeding conditions. After feeding FO, such products are not only found in tumor tissue but are found in normal tissues as well.

The mechanism by which lipid peroxidation products retard or inhibit tumor growth processes is not certain (22). Secondary products of lipid peroxidation (measured by the TBA assay) 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 (23-25). This phenomenon may adversely affect the entering of cells into the cell cycle or it may accelerate their exit from it (i.e. cell death). Furthermore, secondary products of lipid peroxidation can decrease tumor cell survival by inactivating polymerase reactions (26), forming inter- and/or intramolecular linkages between amino acid sulfhydryl groups and biomolecules (DNA, RNA and proteins) (27) and inhibiting polyamine synthesis (28). Such processes may not only result in inhibition of cell proliferation but may also lead to an increase in cell death. Indeed, Gabor and Abraham (4) reported that dietary FO inhibits mammary tumor growth processes by increasing mammary tumor cell death rather than suppressing mammary tumor cell proliferation.

Secondary products of lipid peroxidation, while under certain conditions may decrease tumor growth via a cytostatic/cytolytic mechanism, under other circumstances such products could stimulate tumor growth. For example, a peroxidation product of linoleic acid, i.e., 13-hydroxy (hydroperoxy) octadecadienoate is more mitogenic to

rat colonic mucosa cells (29) or to hamster embryo fibroblasts (30) than is the parent compound. In addition, a peroxidation product of arachidonic acid, i.e. hydroxy (hydroperoxy) eicosatetraenoic acid stimulates rat colonic mucosa cell proliferation while the parent compound itself lacks stimulatory activities (29). Thus, peroxidation products derived from fatty acids in dietary FO (particularly EPA, DHA and possibly linoleic acid) could be stimulatory to tumor growth processes. In the tumor model used in this study (MDA-MB231 human breast carcinoma maintained in athymic nude mice), however, we find no evidence of dietary FO induced stimulation of tumor growth.

The results of our study clearly show that the addition to the FO diet of large amounts of antioxidants (Vit E and TBHQ) significantly counteracted the inhibitory effects of dietary FO on human breast carcinoma growth. In essence, high levels of antioxidants, in the presence of dietary FO, stimulated tumor growth. Other laboratories have provided evidence that supplementation of the diet with antioxidants inhibits tumorigenesis. Clearly dietary antioxidants can suppress carcinogen activation in experimental tumor models (31). Occasionally it is reported that antioxidant supplementation can suppress tumor growth (32,33), although there is considerable evidence to the contrary (34-36). Thus, it is likely that various antioxidants can suppress genotoxic induced initiation and/or progression events in tumorigenesis; evidence that antioxidants can effectively suppress tumor growth processes, however, has not been consistent or compelling.

In conclusion, the results of this study provide evidence that dietary FO can significantly suppress the growth of MDA-MB231 human breast carcinomas maintained in athymic nude mice, even in the presence

of substantial amounts of dietary linoleic acid. The suppressive effect appears to be directly related to the accumulation of TBARS in the tumor tissues.

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Table 1. Diet Composition

Ingredients[a]	Percent[b]
fat[c]	20.00
casein[d]	20.17
dl-methionine	0.35
dextrose	32.18
sucrose	16.09
AIN mineral mix[e]	4.13
AIN vitamin mix[f]	1.18
cellulose[g]	5.90

[a]All ingredients (dry components) were obtained from U.S. Biochemical Co. (Cleveland, OH) except sucrose (ICN Biochemicals, Inc., Costa Mesa, CA).

[b]High fat diets (20% wt/wt) consisted of 20% corn oil or a fish oil (menhaden)/corn oil combination totaling 20%.

[c]Corn oil (tocopherol stripped) was obtained from U.S. Biochemical Co. (Cleveland OH). Fish oil (menhaden) was obtained from Zapata Haynie Corp. (Reedville, VA).

[d]Vitamin free, high nitrogen (14.5%).

[e]The mineral mixture supplied, per kg diet: $CaHPO_4$, 20.65g; NaCl, 3.06 g; potassium citrate, 9.09 g; K_2SO_4, 2.15g; MgO, 991.20 mg; manganous carbonate, 144.55 mg; ferric citrate, 247.8 mg; zinc carbonate, 66.08 mg; cupric carbonate, 12.39 mg; KIO_3, 0.41 mg; sodium selenite, 0.41 mg; chromium potassium sulfate, 22.72 mg; sucrose, 4.87 g.

[f]The vitamin mixture provided, per kg diet: thiamine HCl, 7.08 mg; riboflavin, 708 mg; pyridoxine HCl, 8.26 mg; nicotinic acid, 35.40 mg; D-calcium pantothenate, 18.88 mg; folic acid, 2.36 mg; DL-α-tocopheryl acetate, 236.00 mg; cholecalciferol, 0.03 mg; menaquionine, 0.06 mg; sucrose, 11.48 g.

[g]Celufil, non nutritive bulk.

Table 2. Predominant Fatty Acids (%)

Fatty acids[a]	Corn oil	Fish (menhaden) oil
Myristic (14:0)	---	6.7
Palmitic (16:0)	10.8	15.7
Palmitoleic (16:1)	---	8.7
Stearic (18:0)	2.1	2.7
Oleic (18:1)	26.5	14.3
Linoleic (18:2)	60.0	1.8
Linolenic (18:3)	---	1.5
Arachidonic (20:4)	---	2.2
Eicosapentaenoic (20:5)	---	15.5
Docosahexaenoic (22:6)	---	12.1

[a]Fatty acids concentrations <1% are not included.

Table 3. Effect of different ratios of dietary corn oil and fish oil on the growth and lipid peroxidation product concentrations of MDA-MB231 human breast carcinomas in athymic nude mice.

Diet groups[a]	Mean mouse body weight (g)±S.E.	Number of tumors	Mean tumor volume (cm^3)±S.E.[b]	Mean tumor lipid peroxidation product levels (μg TBARS/g sample)±S.E.[b]
20% corn oil (12.0% linoleic acid)	28.1±0.5	60	2.55±0.31[c]	0.26±0.04[c]
15% corn oil/ 5% fish oil (9.1% linoleic acid)	27.3±0.4	64	1.23±0.15[d]	0.37±0.02[d]
10% corn oil/ 10% fish oil (6.2% linoleic acid)	27.1±0.7	79	1.16±0.61[d]	0.40±0.04[d]
5% corn oil/ 15% fish oil (3.3% linoleic acid)	27.1±0.3	66	0.88±0.11[d]	0.59±0.03[e]

[a]Twenty to 25 animals/group.

[b]Values with different superscript letters in the same column are significantly different at $p<0.05$.

Table 4. Effect of different ratios of dietary corn oil and fish oil and the influence of antioxidants on the growth and lipid peroxidation product concentrations of MDA-MB231 human breast carcinomas in athymic nude mice.

Diet groups[a]	Mean mouse body weight (g)±S.E.	Number of tumors	Mean tumor volume (cm^3)±S.E.[b]	Mean tumor lipid peroxidation product levels (nmol TBARS/mg protein)±S.E.[b]
20% corn oil (12.0% linoleic acid)	30.1±0.7	20	2.36±0.90[c]	1.49±0.04[c]
15% corn oil/5% fish oil (9.1% linoleic acid)	30.7±1.0	25	2.08±0.36[c]	1.66±0.04[d]
10% corn oil/10% fish oil (6.2% linoleic acid)	29.9±0.7	26	0.95±0.14[d]	1.86±0.03[e]
5% corn oil/15% fish oil (3.3% linoleic acid)	28.9±0.6	28	0.72±0.20[d]	1.96±0.03[f]
1% corn oil/19% fish oil (0.9% linoleic acid)	28.6±0.9	24	0.53±0.09[d]	2.02±0.07[f]
1% corn oil/19% fish oil + antioxidants (Vit E 2000 IU/kg and TBHQ 2% total fat) (0.9% linoleic acid)	29.5±0.8	19	1.65±0.30[c]	1.48±0.04[c]

[a]Six to 9 animals/group.
[b]Values with different superscript letters in the same column are significantly different at $p<0.05$.

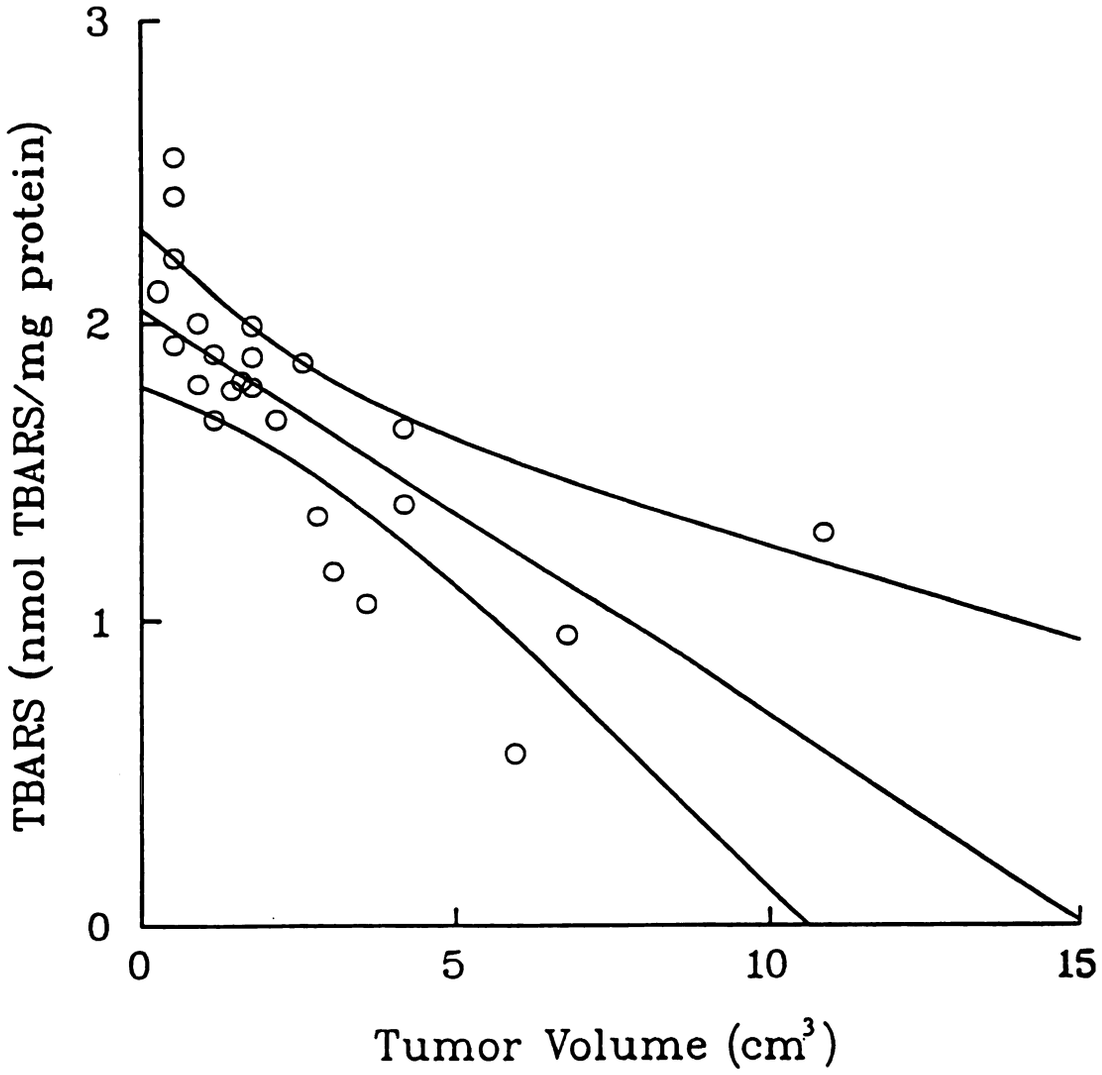
Table 5. Effect of the amount of time the fish oil diet remained in the food jar under usual feeding conditions on the growth and lipid peroxidation product concentrations of MDA-MB231 human breast carcinomas in athymic nude mice.

Length of time diet remained in the food jar[a]	Mean mouse body weight (g)±S.E.[b]	Number of tumors	Mean tumor volume (cm^3)±S.E.[b]	Mean tumor lipid peroxidation product levels (nmol TBARS/mg protein)±S.E.[b]
24 hours (1% corn oil/19% fish oil)	30.6±0.9[c]	24	0.70±0.17[c]	2.04±0.09[c]
48 hours (1% corn oil/19% fish oil)	28.5±0.9	28	0.48±0.11[d]	2.40±0.10[d]
72 hours (1% corn oil/19% fish oil)	27.4±0.5[d]	26	0.25±0.27[e]	3.69±0.14[e]

[a]Eight to 9 animals/group.

[b]Values with different superscript letters in the same column are significantly different at $p < 0.05$

Figure 1. Relationship between mean carcinoma volume (cm^3) and mean carcinoma TBARS concentrations per individual MDA-MB231 human breast carcinoma in athymic nude mice fed a 1% corn oil/19% fish oil diet (N=24). Eight animals in this dietary group. Curved lines represent a 95% confidence interval. Pearson correlation coefficient = 0.725 ($p<0.05$).



Chapter 4

Dietary fish oil induced inhibition of human breast carcinoma growth: a phenomenon of reduced cellular DNA synthesis or increased cell loss

ABSTRACT

Diets high in unsaturated fatty acids, especially those containing high levels of linoleic acid, e.g., corn oil, enhance mammary gland tumorigenesis in experimental animals. In contrast, diets high in long-chain polyunsaturated fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic (DHA), e.g. menhaden oil, appear to have a suppressive effect on this tumorigenic process. Many mechanisms have been proposed to explain the tumor inhibitory action exerted by menhaden oil and other fish oils, e.g., differences in prostaglandin metabolism, energy efficiency, alterations of the immune system, changes in lipid peroxidation, etc. Fundamental to a mechanistic understanding of this phenomenon, however, is an understanding as to whether or not the tumor inhibitory activities of dietary fish oil is mediated via an inhibition of tumor cell proliferation or mediated via an enhancement of tumor cell loss. Whether the amount of dietary fat or the type of fat effects mammary tumorigenic processes, via an effect on tumor cell proliferation or tumor cell loss, has not been clearly established. In the studies described in this communication, three methods were utilized to study tumor cell proliferation, i.e., H^3-thymidine autoradiographic analysis, 5-bromo 2′-deoxyuridine (Brdu) flow cytometric analysis, and proliferative cell nuclear antigen (PCNA) flow cytometric analysis. Two methods were used to study tumor cell loss, i.e., a determination of the I^{125}Urd tumor emission rate and a determination of a cell loss factor from the formulas of Steel and Begg. The tumor examined was the human breast carcinoma cell line MDA-MB231 maintained in athymic nude mouse. No significant difference in cell proliferation between carcinomas of mice fed a high corn oil diet

(20% w/w) and a diet high in fish oil (19% menhaden oil, 1% corn oil). In contrast, a significant ($p<0.05$) increase in the rate of I^{125}Urd emission rate and cell loss factor from the carcinomas in the fish oil fed mice compared to the corn oil fed mice was observed. In summary, the decreased tumor volume in the human breast carcinomas maintained in athymic nude mice fed a fish oil diet as compared to those fed a corn oil diet, appears to be due, at least in part, to an increased rate of carcinoma cell loss rather than a decreased rate of carcinoma cell proliferation.

INTRODUCTION

The mechanism by which diets high in certain fats such as corn oil are capable of enhancing mammary tumorigenesis in rodents (1) and increase human breast carcinoma size in athymic nude mice (2) is unclear. Moreover, the mechanism by which diets high in long-chain polyunsaturated fatty acids (PUFA) such as fish oils, can effectively suppress mammary tumorigenesis in rodents (1) and inhibit human breast carcinoma growth in athymic nude mice (2,3) also remains to be determined. This raises a fundamental question in tumor biology that has not been rigorously examined. How do dietary fats exert their enhancing or suppressive activity at a tumor growth kinetic (tumor cell loss vs tumor cell proliferation) level? Only a few research groups (4-6) have examined cell proliferation in tumors of animals fed high fat diets. One group (7) reports no difference in cell proliferation rates in transplantable mouse mammary tumors from animals fed diets composed of unsaturated vs saturated fats. In another study, Abraham et al. (8) hypothesized that the increase in tumor size induced by a corn oil diet compared to hydrogenated cottonseed oil or fish

(menhaden) oil diet was due to a decrease in cell loss (cell death) as a result of the high corn oil diet impairing immune system activity. In other studies (5,6,9-10), an increase in cell proliferation was observed by an increase in H^3-thymidine incorporation into DNA of carcinogen-induced rat mammary tumors from rats fed a high corn oil diet compared to those fed lower levels of corn oil. They concluded that this increase in mammary tumor growth was due to an increase in carcinoma cell proliferation by providing diets high in corn oil. Our study was designed to determine if the growth of a human breast carcinoma cell line (MDA-MB231) in vivo (athymic nude mice), as a function of feeding high levels of either corn oil or fish oil (menhaden), is due to changes in carcinoma cell proliferation and/or changes in carcinoma cell loss. The knowledge of how dietary fats can affect mammary carcinoma growth dynamics is critical to a mechanistic understanding of nutritional tumorigenesis.

MATERIALS AND METHODS

Animals and Diets

Female athymic nude mice (Harlan Sprague-Dawley Inc., Madison, WI) 4-5 weeks old were used in these experiments. The mice were maintained under aseptic conditions which included an enclosed overhead laminar flow hood and were housed in sterilized cages, with sterilized bedding and provided sterilized drinking water in a temperature-controlled (24°C) and light-controlled (14 h/day) room. Autoclaved laboratory mouse chow (Purina Mills Inc., St. Louis, MO) was fed ad libitum before and until 7-10 days after human breast carcinoma transplantation. Thereafter mice were fed ad libitum purified diets for 4 to 6 weeks (unless indicated otherwise) (Table 1). All dietary ingredients were

obtained from U.S. Biochemicals Inc. (Cleveland, OH) except sucrose, which was obtained from ICN Biochemicals Inc. (Costa Mesa, CA), and fish oil (menhaden), which was obtained from Zapata Haynie Corp. (Reedville, VA). The percentages of predominant fatty acids (1% or greater, manufacturer's specifications) of the dietary oils are shown in Table 2. The diets were prepared weekly and stored at -20°C, individually packed in small plastic sealed bags of sufficient size for one day's feed. Mice were fed daily and non-consumed food discarded daily. Since purified diets were not sterilized, antibiotics (Bacitracin combined with Streptomycin or Neomycin, 1 g/L) were added to the distilled drinking water.

Human Breast Carcinoma Transplantation

Palpable MDA-MB231 human breast carcinomas (American Type Culture Collection, Rockville, MD) were surgically excised from female athymic mice, cut into slices (2x4 mm, 0.1-0.3 mm thick) and implanted into recipient female athymic mice under aseptic conditions. Mice were anesthetized with sodium pentobarbital (60 μg/g, i.p.) prior to transplantation. An incision was made in the integument, the tumor slices were placed s.c. in the dorsum at distances from each other of at least 2 cm, 3 to 4 slices/mouse (autoradiograph and flow cytometer experiments). One slice per mouse was placed in the middle of the upper back between the shoulder blades in the animals used for the cell loss experiments. The carcinoma grafts were established in the host animals before the onset of experimental dietary treatments.

Preparation of Tumor Tissue Slices for Histology and Autoradiography

After being fed the diet for 5 to 6 weeks mice were sacrificed. The tumors were excised and cut into slices (1-2 mm). Tumor slices

were incubated in 10x30 mm Falcon disposable Petri dishes (2 slices/dish) containing 2.5 ml of medium (10X Waysmouth MB 752/1 medium, GIBCO Labs, Grand Island, NY). Per 100 ml of media, the following constituents were added: 35 mg glutamine, 3.5 mg penicillin and 125 mg of sodium bicarbonate. Sterile H^3-thymidine (45 Ci/nmol, New England Nuclear, Boston, MA) was added at a concentration of 1 μCi/ml of medium. The Petri dishes were placed in a small gassing chamber, housed in an incubator at 37°C. The chamber was continuously infused with gas 95% O_2: 5% CO_2 for a 4 hr incubation period. The slices were then fixed in Bouins Fluid, embedded in a paraffin preparation (Tissue-prep, Fisher Scientific Co., Fairlawn, NJ), sectioned at 5-7 μm and mounted on glass slides. Two series of tissue sections were prepared; one series was stained with hematoxylin and eosin (H & E) and the other series was used for the autoradiographs. The slides for autoradiography were dipped in NTB2 nuclear tract emulsion (Eastman Kodak Co., Rochester, NY), dried and stored away from light in tight black boxes with a desiccant for 14 days at 4°C. After two weeks, the slides were developed and stained by H & E using a standard method (11). The slides were then coded (identity of treatment unknown) and the number of H^3-thymidine labelled breast carcinoma cells per area was computed for each carcinoma of both dietary groups. Group mean differences between labelled cells were evaluated statistically by the students t-test.

Flow Cytometric Analysis of Human Breast Carcinomas for DNA Synthesis: Bromodeoxyuridine (Brdu) Technique

Preparation and Dissociation of Tumor Tissue

One hour prior to sacrifice, mice were injected i.p. with 5-bromo 2'-deoxyuridine (Brdu) at a concentration of 50 mg/kg body weight (Sigma Chemical Co., St. Louis, MO). After 1 hr, animals were terminated by an overdose of CO_2 and the tumors excised. Necrotic tissue was trimmed from the tumor and a 7 mm biopsy punch was used to obtain a tumor sample. The sample was minced with a single-edged razor blade and placed in a 12x75 mm glass tube containing 2 ml of ice cold 70% ETOH. The tubes were then sealed with parafilm and stored at -20°C until the dissociation step. Tissue samples were removed from -20°C storage and approximately 40 mg of tissue was finely minced with a scalpel or single-edged razor blade. The tissue was then simultaneously dissociated and denatured by placing in a 25 ml Erlenmeyer flask containing 2 ml of 0.4 mg/ml pepsin in 2 N HCl. The flasks were placed in a shaking water bath at room temperature for 1.5 to 2 hours or until the cells could be easily dispersed by gentle up and down pipetting with a pasteur pipette. The cell suspensions were then filtered through a 50 μg mesh and washed twice with 2 ml of PBS (pH 7.4) containing 0.1% BSA and 0.05% Tween 20 (PBT buffer).

Staining the Cells with Anti-Brdu and Propidium Iodide

Approximately $5x10^5$ to $1x10^6$ cells were resuspended in 100 μl of PBT buffer containing 2 μg/ml of anti-Brdu antibody (Boehringer-Mannheim Co., Indianapolis, IN). The tubes were incubated for 30 min at room temperature, washed with 2 ml of PBT buffer and resuspended in 100 μl of PBS containing 10 μg/ml goat antimouse IgG-FITC. The tubes

were incubated for 30 min at room temperature, washed twice with PBS and resuspended in 1 ml of PBS containing 10 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO). A control for non-specific binding was run for each sample by preparing a duplicate tube with no anti-Brdu. The tubes were incubated overnight at 4°C and analyzed using a flow cytometer (Ortho 50H, Ortho Diagnostics, Westward, MA). The amount of Brdu uptake was reported as the percent of cells with green fluorescence intensity above that of the non-specific binding control. Mice that were not injected with Brdu were used as a control to eliminate background fluorescence.

Cell Preparation for Anti-Proliferative Cell Nuclear Antigen (PCNA) Assay

The cold 70% ethanol-fixed tumors prepared for the Brdu assay were also used for the PCNA assay. The tissue samples were removed from the 70% ethanol, rinsed and placed in an Erlenmeyer flask containing 2 ml of pepsin (0.4 mg/ml in 0.1N HCl). Dissociation was carried out on a shaking water bath at room temperature for 30-60 min or until the cells were easily dispersed by gentle up and down pipetting with a Pasteur pipette. Cells were washed twice in PBS (pH 7.4) containing 0.1% Triton-X 100. Cells were then suspended in 100 μl PBS containing 25 μg/ml of PCNA (Boehringer-Mannheim Co., Indianapolis, IN) and 0.1% BSA. Cells were then incubated at room temperature for 30 min. Cells were washed and resuspended in 100 μl of PBS containing 10 μg/ml goat anti-mouse FITC. Tubes were incubated for 30 min at room temperature, washed twice with PBS and resuspended in 1 ml of PBS containing 10 μg/ml of propidium iodide. The cold 70% ethanol-fixed tumors prepared for the Brdu assay were also used for the PCNA assay.

The tissue samples were removed from the 70% ethanol, minced and placed in an Erlenmeyer flask containing 2 ml of pepsin (0.4 mg/ml in 0.1N HCl). Dissociation was carried out on a shaking water bath at room temperature for 30-60 min or until the cells were easily dispersed by gentle up and down pipetting with a pasteur pipette. An IgG_1 antibody of irrelevant specificity was used as control to monitor non-specific binding. The cells were analyzed using a flow cytometer (Ortho 50H, Ortho Diagnostics, Westward, MA). The amount of PCNA was reported as percentage of green fluorescent cells.

Assessment of Cell Loss in Human Breast Carcinomas

Cell loss is defined as the rate of loss of cells as a fraction of the rate at which cells are being added to the tumor volume by cell proliferation. Cell loss is an important factor in estimating the growth potential of a tumor (12). In order to facilitate the study of this phenomenon in our experimental model, we proceeded as follows. The human breast carcinomas, maintained in athymic nude mice, were measured weekly with a Vernier caliper. The weekly increase in volume (cm^3) was determined for each carcinoma. After the mice had been fed diet for 6 weeks, the carcinoma-bearing athymic nude mice were injected i.p. with 5 μCi of I^{125}-iodo 2'-deoxyuridine (I^{125}Urd, 6 mCi/mg, Sigma Chemical Co., St. Louis, MO). In order to prevent excess concentration of I^{125} in the thyroid, each mouse was given 0.1% KI in the drinking water commencing 3 days prior to I^{125}Urd administration. Twenty-four hours after I^{125}Urd injection, mice were lightly anesthetized with ether and secured in a holding apparatus to allow for gamma emission readings. Emissions were read using a Geiger counter with a

NaI crystal, 2 inch diameter and 0.04 inches thick, Model leg-1, low energy gamma probe, 61% efficiency, Eberline Inc., Santa Fe, NM. Care was taken to place the probe in an identical position on top of the carcinoma in contact with the integument overlying the outer surface. Duplicate 1 minute emission readings (cpm) were recorded for each carcinoma for seven consecutive days, subtracting background emissions. Mean rate of I^{125}Urd loss from each tumor was calculated as follows:

$y=mx+b$ y=natural log of the daily mean I^{125}Urd emissions (from duplicate measurements) (cpm)-background emissions (cpm)

m=slope (K_L, rate constant)

x=time (days)

b=y intercept=activity at time zero

Using the above equation, a graph was generated for each carcinoma as follows:

ln[cpm(1-7)-cpm(0)] vs time (days), ln=natural log

cpm(1-7)=counts per minute (emissions) from day 1 to day 7

cpm(0)=counts per minute (emissions) at day 0

The resulting slope, or rate constant K_L, was utilized to compute carcinoma cell loss factor using the following formulas:

$$\phi=\frac{T_D}{(T_{1/2}+T_D)}$$ Begg's Formula (13)

ϕ=cell loss factor=cell loss rate expressed as percent of the cell birth rate

T_D=tumor doubling time in days (calculated by determining the number of days for tumor to double in size)

$T_{1/2}$=time (days) for I^{125}Urd emission from the tumor to reach 1/2 of initial (time 0) emission rate

$\phi = 1 - \frac{T_P}{T_D}$ Steel's Formula (12)

ϕ=cell loss factor=cell loss rate expressed as percent of the cell birth rate

T_D=tumor doubling time in days (calculated by determining the number of days for tumor to double in size)

$T_p = \frac{\ln 2}{K_L}$

Principles for assessing cell loss from growing tumors in situ using these formulas have been validated by Kallman et al. (14).

RESULTS

In Table 3 after mice were fed corn oil (CO) and fish oil (FO) diets for a period of only one week (Study 1), the difference in mean tumor volumes did not reach a level of 5% significance. Also no significant difference between mice fed CO and FO diets was obtained in DNA synthesis parameters (H^3-thymidine autoradiograph analysis and Brdu flow cytometry analyses). This trend was also observed in tumors of animals fed CO and FO diets for a period of two weeks (Study 2), in which mean tumor volumes and mean tumor DNA synthesis parameters were not significantly different. The animals fed the CO diet for four weeks (Study 3) had a significantly larger ($p<0.05$) tumor volume than those fed a FO diet; nevertheless no significant difference was detected in mean H^3-thymidine autoradiograph analysis. In another study in which animals were fed diets for 4 weeks (Study 4) we also observed a significantly larger tumor volume ($p<0.05$) in the CO fed animals compared to those fed FO. However, when FO was supplemented with excess antioxidants, mean tumor volume of animals fed the supplemented FO was comparable to the mean tumor volume of the CO fed

animals. Again no significant difference was observed in tumor DNA synthesis parameters. In addition, animals fed FO supplemented with iron, mean tumor volume were significantly less compared to the other three experimental groups (CO, FO, FO+antioxidants) but no significant difference in mean tumor H^3-thymidine analysis was detected. After feeding CO and FO diets for six weeks (Study 5), CO fed animals had a significantly larger ($p<0.05$) mean tumor volume than those fed FO. However, no significant difference in tumor DNA synthesis parameters was observed. After feeding diets for 10 weeks (Study 6), a significant difference in mean tumor volume was not reached, neither was a significant difference obtained in tumor DNA synthesis parameters.

In Table 4 (Study 7), after mice were fed a CO and FO diet for six weeks, animals fed a CO diet had a significantly larger ($p<0.05$) tumor volume compared to those fed FO but not compared to those fed the antioxidant supplemented FO diet. No significant difference in tumor DNA synthesis parameters (Brdu analysis and PCNA analysis) was detected between these three dietary groups.

In Table 5 (Study 8), after feeding different ratios of CO and FO for a period of six weeks the animals fed 15% CO/5% FO had a significantly larger ($p<0.05$) tumor volume than the ones fed 10% CO/10% FO and 5% CO/15% FO. The animals fed 10% CO/10% FO had a higher tumor volume than those fed 5% CO/15% FO but this difference did not reach a level of 5% significance. Also no significant difference was detected in tumor mean Brdu analysis between these three groups.

In Table 6 (Study 9), after feeding tumor bearing mice a CO and FO diet for two weeks no significant difference was detected in mean tumor

volume, mean rate of I^{125}Urd loss from tumors nor mean tumor cell loss factors between the two diet groups. In Study 10, after feeding a CO and FO diet for four weeks, no significant difference was detected in mean tumor volume, mean rate of I^{125}Urd loss from tumors, nor mean tumor cell loss factors. In Study 11, after feeding a CO and FO diet for six weeks the CO fed animals had a significantly larger ($p<0.05$) mean tumor volume compared to the FO fed animals; the tumor volume in the CO fed animals, however, was not significantly different from that observed in the antioxidant supplemented FO fed animals. Mean rate of I^{125}Urd loss from tumors was significantly ($p<0.05$) lower in the CO fed animals compared to the FO fed animals and the antioxidant supplemented FO group. Mean tumor cell loss factor as determined by Steel (12) and Begg (13) was also significantly lower in the CO fed group compared to the FO fed group and to the antioxidant supplemented FO group.

Figure 1 compares the mean slopes of the rate of I^{125}Urd loss from the tumors of the three dietary groups (CO, FO+A and FO). A significant difference ($p<0.05$) in slopes between CO and FO and CO and FO+A was observed; the slopes of the FO and FO+A dietary groups were virtually identical.

DISCUSSION

Diets high in polyunsaturated fatty acids (e.g., corn oil), when fed to rodents, causes an increase in size and number of mammary tumors when compared to rodents fed low levels of the same fat or high levels of other types of fat (e.g., beef tallow and certain fish oils) (15). To examine the tumor growth kinetics of this phenomenon, only a few laboratories (4-8) have investigated the differential effects of

dietary fat on tumor cell proliferation or on tumor cell loss (cytolysis). Abraham et al. (8) reported a significantly smaller tumor size of transplantable mouse mammary tumors in mice fed a high FO diet compared to tumors of mice fed a high CO diet. They accounted for this result by providing data of an increase cell loss in tumors of rodents fed a high FO diet compared to the cell loss obtained from those tumors of mice fed a high CO diet. Previously they reported (4,7) no significant difference in tumor cell proliferation parameters when feeding diets high in unsaturated and saturated fatty acids to mammary tumor bearing rodents in spite of obtaining a significant difference in tumor size. When examining different levels of dietary fat, Oyaizu et al. (5) and Noguchi and colleagues (6,9,10) reported a smaller mammary tumor size in rats fed a low level of CO compared to rats fed a high level of CO; a decrease in carcinoma cell proliferation parameters in the tumors of rats fed the low level CO diet compared to those fed the high level of CO was observed. Clearly, more studies are required to have a more definitive understanding as to the effect of the type and amount of dietary fat on tumor cell proliferation dynamics in mammary tumors in order to have a better understanding of the nutritional influence on tumor growth processes.

Thus, an important question remains unanswered. Is the decreased size of tumors of animals fed high FO diets due to a decrease in DNA synthesis, or because of an increase in cell loss (cytolysis)? The H^3-thymidine autoradiographic methodology is a precise means to assess DNA synthesis (16). Furthermore, this technique is an effective means of providing a quantitative differentiation between carcinoma cell and stromal cell proliferation processes (17). One drawback of the

H^3-thymidine autoradiographic methodology is that it is extremely time consuming. On the other hand, the flow cytometry method of measuring DNA synthesis provides a fast (labeling and detection can be performed the same day), sensitive and quantitative way to measure DNA synthesis in suspended cells. Brdu is an analog of thymidine that is concentrated only in cells in active DNA synthesis (18). Quantitation of Brdu concentration in DNA is made possible by the development of a monoclonal antibody against Brdu (19). The H^3-thymidine autoradiographic technique and the Brdu flow cytometric technique, as methods of estimating cell proliferation have been reported to be in close agreement with each other (20). The PCNA flow cytometric technique has also been used to study cell proliferation processes. PCNA possesses a temporal specificity which makes it a suitable marker for cell proliferation. PCNA begins to accumulate during the G_1 phase of the cell cycle, is most abundant during the S phase and declines during G2/M phase (21). PCNA has been successfully used to selectively identify proliferating cells in solid tumors (22).

The method for the assessment of cell loss from growing tumors was originally described by Steel (12) and validated by Begg (13) and Kallman et al. (14). Steel's cell loss factor measures the rate of loss of cells as a fraction of the rate at which cells are being added to the tumor volume by cell proliferation (23). This factor, therefore, expresses the growth potential of a tumor (ratio of cell loss rate to the cell birth rate). The extent to which processes of cell loss are competing with the process of cell proliferation can be obtained utilizing the formula:

$\phi = 1 \frac{T_P}{T_D}$, where ϕ=the cell loss factor.

A modified version of Steel's cell loss measurement concept was first utilized in a dietary study of tumor growth by Abraham and colleagues (18). The method of measuring cell loss from tumors in situ by using the I^{125} deoxyuridine (I^{125}Urd) technique was first described by Begg (13). Begg's derived tumor kinetic parameters originated from Steel's formula for cell loss factor (11). Begg equates the I^{125}Urd emission rate (K_L, loss of radioactivity) to the rate of cell loss. The slope of the I^{125}Urd emission rate in a semilogarithmic plot is defined as $T_{1/2}$ (time to halve the radioactivity). The modified adaptation of Steel's formula is:

$\phi = T_D/(T_{1/2}+T_D)$.

The pragmatic difference between these two formulas is that Begg's derived formula takes into account only one point (point of 1/2 radioactivity) on the curve generated by the I^{125}Urd emission data, whereas Steel's formula takes into account the total curve generated by the I^{125} emission data. Therefore, by utilizing the whole curve, Steel's formula provides a more precise assessment of cell loss factor. In general, both formulas were in close agreement in these studies. These techniques to measure cell loss are very attractive since they provide a direct determination, and therefore, are superior to methods which depend solely on calculated and measured doubling times of tumor growth. Another advantage of these methods is that they require fewer animals than methods requiring the excision of tumors, since each animal contributes several time points. In addition, variation is

reduced since each tumor acts as its own control. Moreover, it allows for cell loss rates of individual tumors to be determined. Currently, the I^{125}Urd technique offers the only direct non-invasive method of assessing cell loss in individual tumors. Although errors of the in situ technique are smaller and less frequent than those occurring with other methods, the problems of reutilization of the isotope are still present. The isotope could also be trapped in necrotic areas inside or surrounding the tumor. For this reason, in our experiments, tumors with overt necrotic areas were not used. Since our cell lines do not elicit any substantial immune response, in athymic nude mice, we did not have the problem of additional necrosis induced by immune cell infiltration of the tumor tissue. Thus, our experimental model utilizing athymic nude mice bearing human breast carcinoma cell lines is suitable for determination of the cell loss factor by the I^{125}Urd in situ technique. In addition to reporting Steel's and Begg's cell loss factor, we report the tumor I^{125}Urd emission rate which, indirectly can be equated to cell loss rate.

No significant differences in DNA synthesis parameters between the diet groups (CO and FO) were observed (Studies 1-6, in Table 3, Study 7 in Table 4 and Study 8 in Table 5), despite the significant differences in tumor size that were observed in a number of these studies (Studies 3-5,7,8). The small numerical decrease in DNA synthesis parameters in tumors of FO fed mice (3-5% decrease) may have relevant biological significance. This very small decrease in DNA synthesis may prove of importance, if this difference is real and can be extended throughout the entire dietary feeding period.

In Study 4 (Table 3), two additional FO groups were added, an antioxidant supplemented FO group (FO+A), and an iron-supplemented FO group (FO+I). In this study, supplementation with antioxidants significantly enhanced tumor volume of the FO fed animals, while in contrast, supplementation with iron significantly decreased tumor volume of the FO fed animals. This appears to be due to differences in cytostatic/cytolytic lipid peroxidation product accumulation in the tumors as we reported previously (2,3). Nevertheless, in spite of this tumor volume difference, no significant difference in cell proliferation parameters in the tumors was observed. Study 7 (Table 4) followed a similar trend as Study 4 in which a significant difference was observed in mean tumor volume between the CO and the FO fed groups, with the antioxidant supplemented FO having a comparable tumor volume to the CO fed group; no significant difference in cell proliferation parameters was once again observed. In Study 8 (Table 5), different ratios of CO and FO were fed which resulted in an inverse relationship in which a decreasing tumor volume was evident as the FO content of the diet increased; once again no significant difference in cell proliferation parameters was observed. These results are in accordance with those of Abraham et al. (4,7,8) in which the difference in mammary tumor size between CO and FO fed animals cannot be accounted for by the fraction of tumor cells that were actively proliferating.

Our results suggest that parameters other than cell proliferation may be the primary mechanism by which differences in tumor volume between dietary CO and FO fed animals is achieved. Studies 9,10,11 (Table 6) furnish a possible answer to the mechanistic question of how dietary FO affects tumor growth-related kinetic parameters by providing

yet another parameter that can substantially influence the size of tumors; this parameter is cell loss. Cell loss is a biological phenomenon that occurs in normal and neoplastic tissue and is an important kinetic variable which can determine the dynamic state of tissue growth (19). Importantly, when a significant difference in tumor volume was not reached between the CO and FO dietary groups, no significant difference was observed in tumor cell loss parameters (I^{125}Urd emission rate and cell loss factors, Studies 9,10). In contrast, when a significant difference was found in mean tumor volume, a significant difference was also observed in tumor cell loss parameters (Study 11). These studies are in agreement with those of Abraham et al. (8) in which a significant difference in mammary tumor size, in CO and FO fed animals, was accompanied by a significant difference in tumor cell loss; mammary tumor cell loss was greatest in the FO fed animals. The addition of antioxidants to a dietary FO group did not have a significant effect on tumor volume compared to either of the two other dietary groups (CO and FO) in Study 11. Nevertheless, in Study 4 and 7, a significant increase in tumor volume was obtained when feeding the FO+A diet compared to the group fed solely FO. Study 4 and 7 are in agreement with previous reports from our laboratory (2,3). Also in Study 11, no difference in tumor cell loss parameters was obtained between FO+A and the FO group. The reason for this is not known. It is conceivable that the FO used in Study 11 could have been substantially oxidized prior to diet preparation. The already excessively oxidized FO could prevent any substantial antioxidative effect by the addition of antioxidants. This would result in similar tumor volumes and cell loss parameters in animals fed FO and FO+A

diets. These results, albeit preliminary, suggest that differences in mammary tumor cell loss parameters in CO and FO fed animals are very important, perhaps more important than tumor cell proliferation, in determining the extent of volume of these tumors. More studies are needed to confirm these preliminary results in order to provide a conclusive unifying concept to explain how dietary fat affects tumor growth. Nevertheless, these experiments and those reported earlier (2,3) support the concept that FO suppresses human breast carcinoma growth in athymic nude mice by increasing the concentration of secondary products of lipid peroxidation in the tumor; such products (cytostatic/cytolytic) significantly increase tumor cell loss.

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Table 1. Diet composition.

Ingredient[1]	Amount
	g/100 g diet
Fat[2]	20.00
Casein[3]	20.17
DL-Methionine	0.35
Dextrose	32.18
Sucrose	16.09
AIN Mineral mix[4]	4.13
AIN Vitamin mix[4]	1.18
Cellulose[5]	5.90

[1]All ingredients (dry components) were obtained from U.S. Biochemical (Cleveland, OH) except sucrose (ICN Biochemicals, Costa Mesa, CA).

[2]High-fat diets (20% wt/wt) contained 20% corn oil or 19% fish oil (menhaden) + 1% corn oil. Menhaden oil contains 0.03 g/kg of all-rac -α-tocopherol. Corn oil (tocopherol stripped) was obtained from U.S. Biochemical. Fish oil (menhaden) was obtained from Zapata Haynie (Reedville, VA).

[3]Vitamin free, high nitrogen (14.5%).

[4]AIN (1977).

[5]Celufil, non-nutritive bulk.

Table 2. Predominant fatty acids in oils and fats (percentage)

Fatty acids[a]	Corn oil	Menhaden oil
Caprylic (8:0)	-	-
Capric (10:0)	-	-
Lauric (12:0)	-	-
Myristic (14:0)	-	8.0
Palmitic (16:0)	10.1	28.9
Palmitoleic (16:1)	-	7.9
Stearic (18:0)	1.6	4.0
Oleic (18:1)	31.4	13.4
Linoleic (18:2)	56.3	1.1
Linolenic (18:3)	-	1.0
Eicosapentaenoic (20.5)	-	10.2
Docosahexaenoic (22.6)	-	12.8

[a]Fatty acid concentrations less than 1% are not included.

Table 3. Effect of dietary fat (corn oil and fish oil) on DNA synthesis (H^3-thymidine autoradiography and Brdu) of human breast carcinoma MDA-MB231 maintained in athymic nude mice.

Diet	Number of tumors	Mean tumor volume (cm^3±S.E.)	Mean H^3-thymidine autoradiographs (# labeled tumors cells/mm^2 of tissue±S.E.)[e]	Brdu labeling-index (% tumor cells showing Brdu uptake ±S.E.)[e]
1 week on diet (Study 1)				
Corn oil[a]	15	0.12±0.03	n.d.	20.96±1.69(15)
Fish oil[b]	16	0.09±0.03	n.d.	19.85±1.93(16)
2 weeks on diet (Study 2)				
Corn oil[a]	15	0.12±0.03	138.01±12.18(15)	7.26±1.30(14)
Fish oil[b]	9	0.07±0.02	134.70±15.07(9)	10.42±1.92(9)
4 weeks on diet (Study 3)				
Corn oil[a]	55	1.24±0.11[f]	43.54±2.40(55)	n.d.
Fish oil[b]	40	0.60±0.07[g]	41.60±2.49(40)	n.d.
4 weeks on diet plus diet supplementation (Study 4)				
Corn oil[a]	25	1.50±0.15[f]	45.36±2.98(25)	16.65±2.19(25)
Fish oil[b]	21	0.91±0.10[g]	43.19±2.47(21)	11.64±1.17(21)
Fish oil + antioxidants[c]	17	1.48±0.29[f]	45.46±4.63(17)	13.88±1.25(17)
Fish oil + iron[d]	10	0.24±0.07[h]	43.86±5.83(10)	n.d.
6 weeks on diet (Study 5)				
Corn oil[a]	50	1.55±0.16[f]	28.52±2.04	14.17±0.85(42)
Fish oil[b]	55	0.84±0.08[g]	25.89±1.82	12.76±0.80(33)
10 weeks on diet (Study 6)				
Corn oil[a]	8	1.20±0.34	45.79±6.29(8)	4.31±1.11(6)
Fish oil[b]	9	1.19±0.23	40.54±3.80(9)	4.30±0.83(9)

[a]20% corn oil.
[b]19% menhaden oil/1% corn oil.
[c]19% menhaden oil/1% corn oil + alpha tocopherol acetate (8 g/kg diet), tertiary butyl hydroquinone (4 g/kg diet).
[d]19% menhaden oil/1% corn oil + ferric citrate (3 g/kg diet)
[e]number of tumors examined in parenthesis.
[f/g/h]$p<0.05$.

Table 4. Effect of dietary fat (corn oil and fish oil) on DNA synthesis (Brdu and PCNA) of human breast carcinoma MDA-MB231 maintained in athymic nude mice.

Diet 6 weeks on diet (Study 7)	Number of tumors	Mean tumor volume (cm^3 ± S.E.)	Brdu labeling-index (% tumor cells showing Brdu uptake±S.E.)[d]	PCNA labeling-index (% tumor cells PCNA positive ±S.E.)[d]
Corn oil[a]	24	1.40±0.21[e]	4.02±0.52 (24)	14.17±0.85 (24)
Fish oil[b]	18	0.43±0.11[f]	2.63±0.38 (18)	12.76±0.80 (18)
Fish oil + antioxidants[c]	7	1.03±0.18[e]	2.14±0.47 (7)	17.47±2.86 (7)

[a]20% corn oil.

[b]19% menhaden oil/1% corn oil.

[c]19% menhaden oil/1% corn oil + alpha tocopherol acetate (8 g/kg diet) and tertiary butyl hydroquinone (4 g/kg diet).

[d]number of tumors examined in parenthesis.

[e/f]$p<0.05$.

Table 5. Effect of different ratios of dietary fats (corn oil and fish oil) on DNA synthesis (Brdu) of human breast carcinoma MDA-MB231 maintained in athymic nude mice.

Diet 6 weeks on diet (Study 8)	Number of tumors	Mean tumor volume (cm^3 ± S.E.)	Brdu labeling-index (% tumor cells showing Brdu uptake±S.E.)[b]
Corn oil 15%/ fish oil[a] 5%	20	1.24±0.23[c]	10.11±1.55(16)
Corn oil 10%/ fish oil[a] 10%	18	0.65±0.15[d]	7.50±1.09(17)
Corn oil 5%/ fish oil[a] 15%	17	0.34±0.08[d]	6.21±1.30(9)

[a]menhaden oil.

[b]number of tumors examined in parenthesis.

[c/d]$p<0.05$.

Table 6. Effect of dietary fat (corn oil and fish oil) on loss of cells in human breast carcinoma MDA-MB231 maintained in athymic nude mice.

Diet	Number of tumors	Mean tumor volume (cm^3 ± S.E.)	Mean rate of I^{125}Urd loss from tumors (K_L/day ± S.E.)[d,g]	Mean tumor cell loss factor ($\phi = 1 - \frac{T_P}{T_D}$ ± S.E.)[e,g]	Mean tumor cell loss factor [$\phi = T_D/(T_{\frac{1}{2}} + T_D)$ ± S.E.][f,g]
2 weeks on diet (Study 9)					
Corn oil[a]	8	0.48±0.08	0.065±0.01 (8)	1.13±1.12 (8)	6.37±6.37 (8)
Fish oil[b]	6	0.32±0.10	0.095±0.01 (6)	4.38±3.04 (6)	13.75±9.00 (6)
4 weeks on diet (Study 10)					
Corn oil[a]	13	0.33±0.08	0.094±0.01(12)	60.33±4.61(12)	70.26±4.25(12)
Fish oil[b]	13	0.20±0.04	0.138±0.02(11)	64.64±4.85(11)	73.70±4.47(11)
6 weeks on diet (Study 11)					
Corn oil[a]	52	1.55±0.16[h]	0.174±0.01(52)[h]	58.63±3.10(52)[h]	67.58±3.20(52)[h]
Fish oil[b]	45	0.62±0.10[i]	0.224±0.01(45)[i]	66.47±3.16(45)[i]	76.70±3.05(45)[i]
Fish oil + antioxidants[c]	14	1.02±0.13	0.223±0.02(14)[i]	66.43±5.42(14)[i]	76.45±5.81(14)[i]

[a] 20% corn oil.

[b] 19% menhaden oil/1% corn oil.

[c] 19% menhaden oil/1% corn oil + alpha tocopherol acetate (8 g/kg diet) and tertiary butyl hydroquinone (4 g/kg diet).

[d] K_L, mean rate constant of I^{125}Urd loss (ln^2/slope) per day.

[e] Cell loss factor formula as developed by G.G. Steel (Ref. 6).

[f] Cell loss factor formula as developed by A.C. Begg (Ref. 7).

[g] Number of tumors examined in parenthesis.

[h/i] $p<0.05$.

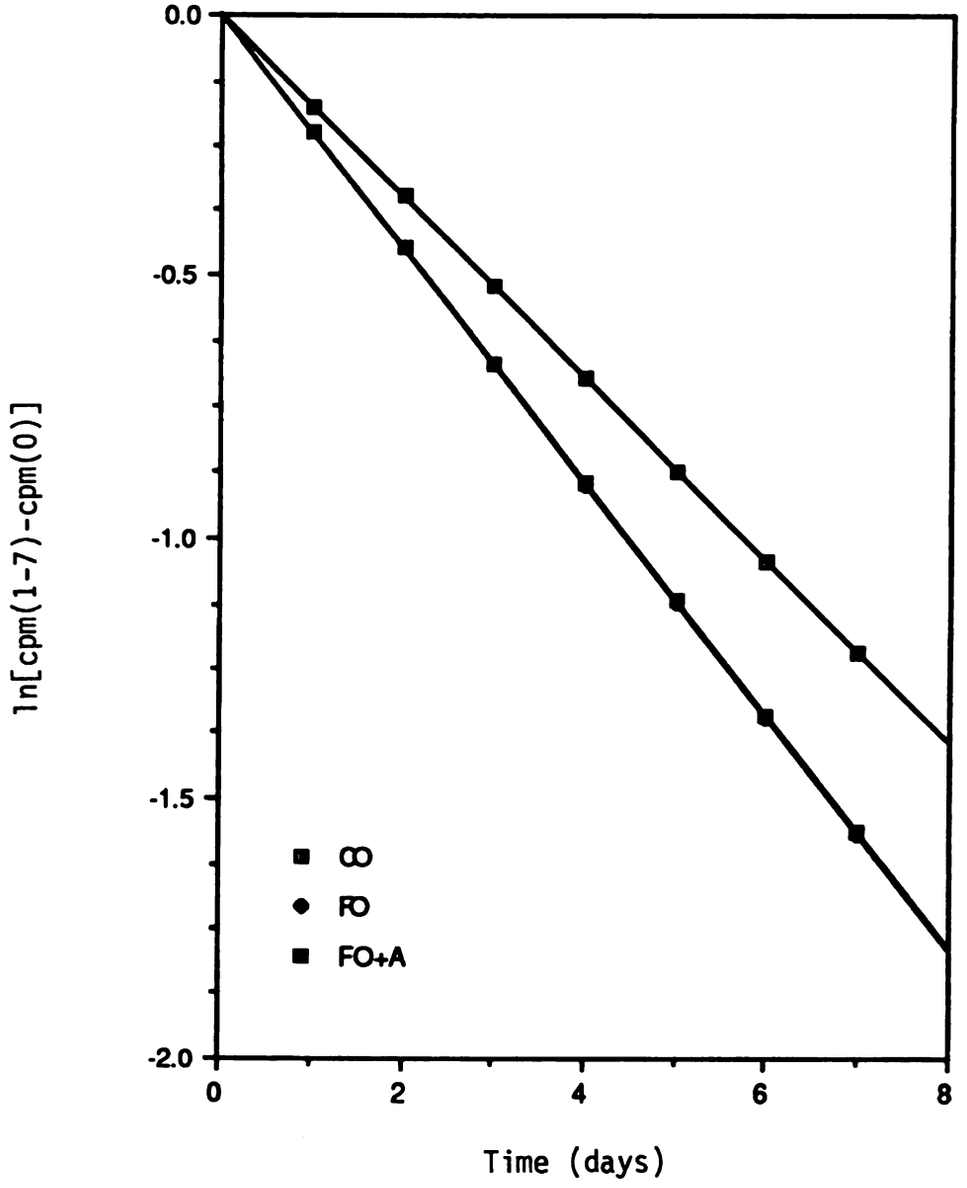

Figure 1. Rate of I^{125}Urd loss from human breast carcinomas in athymic nude mice fed corn oil (CO), fish oil (FO) and fish oil supplemented with antioxidants (FO+A) for a period of 6 weeks. Rates were 0.174±-0.01 (N=52), 0.224±0.01 (N=45) and 0.223±0.02 (N=14) for CO, FO and FO+A, respectively, $p<0.05$.

SUMMARY

It is clear that diets high in omega-3 PUFAs suppress the development and/or growth of mammary gland tumors in experimental animals compared to omega 6-PUFAs. Long chain omega-3 PUFA as EPA and DHA appear to be more effective suppressors of mammary tumorigenesis than shorter chain omega-6 PUFAs such as linoleic acid, although more work is needed to be done to totally verify this point. It is apparent in the athymic nude mice bearing human breast carcinoma MDA-MB231 the omega-3 fatty acids present in menhaden oil have a suppressive effect on tumor growth although various mechanisms may be operative in this suppressive action exerted by the fish oils upon tumor growth. We propose yet another mechanism that has been neglected and that, at least in part, may explain this phenomenon, i.e., that long chain omega-3 polyunsaturated fatty acids may inhibit mammary gland tumorigenesis via a generation and/or accumulation of lipid peroxidation products in the tumor tissue. Also evidence is provided that in the preparation of fish oil diets, the addition of antioxidants at recommended and substantially higher levels does not completely suppress the oxidative damage occurring in experimental diets.

In terms of tumor growth kinetics we studied the effect of dietary fat (corn oil and fish oil) on tumor cell proliferation and tumor cell loss. Preliminary results suggest a higher tumor cell loss occurring in the human breast carcinomas of athymic mice fed fish oil diets

compared to those fed corn oil diets. No significant difference was detected in tumor cell proliferation between these two groups. This increase in cell loss may partly explain why tumor growth is suppressed in animals fed a high fish oil diet compared to those fed high corn oil diets. It is conceivable that this increase in cell loss in the tumors of mice fed fish oil is due to an increase in cytolytic secondary products of lipid peroxidation in the tumor tissue.

CONCLUSIONS

1. Compared to athymic nude mice fed a corn oil diet, athymic nude mice fed fish oil (menhaden) exhibited a significant decrease in growth of human breast carcinomas (MCF-7, MDA-MB231).
2. Human breast carcinomas from athymic nude mice fed fish oil diets contained at least 3 x the level of lipid peroxidation products as that measured in carcinomas obtained from mice fed corn oil diets.
3. Addition of antioxidants (vitamin E and TBHQ) to the fish oil diets significantly reduced the level of lipid peroxidation products in human breast carcinomas and concurrently significantly increased carcinoma growth.
4. Concentrations of lipid peroxidation products in diets high in fish oil, under normal feeding conditions, increase substantially as a function of time (0 hours to 72 hours).
5. The American Institute of Nutrition (AIN) recommended level of addition of a synthetic antioxidant (0.02% w/w of oil) to diets containing high levels of fish oil are insufficient to completely inhibit the accumulation of oxidative metabolites in such diets.
6. TBARS concentrations of organs (liver, heart, kidney, mammary gland, skeletal muscle) and whole body carcass of mice fed fish oil were consistently higher than those in organs and bodies of mice fed corn oil.

7. Diets containing fish oil, with substantial amounts of linoleic acid (up to 9.1% of diet), still suppressed growth of MDA-MB231 human breast carcinomas in athymic nude mice.
8. Increasing the fish oil content of the diet decreased human breast carcinomas (MDA-MB231) growth in athymic nude mice.
9. As the fish oil content of the diet was increased, there was a concurrent increase in human breast carcinoma TBARS concentration.
10. Human breast carcinomas that have the highest concentrations of TBARS have the least tumor growth as measured in tumor volume.
11. Preliminary results suggest that cell loss from tumors has a more important role in tumor growth than does tumor cell proliferation.
12. The decrease in mammary tumor development and/or growth reported in animals fed high levels of fish oil may be due, at least in part, to an accumulation of secondary peroxidation products in the tumor tissue which can probably increase tumor cell loss.

RECOMMENDATIONS

The field of nutritional tumorigenesis has a potential for producing an enormous impact in many areas of cancer research; in relation to nutritionally relevant fatty acids such as linoleic acid and eicosapentaenoic acid, it has been recently reported an apparent regulatory function of these fatty acids on gene expression in vitro (1).

The proposed mechanisms by which diets high in omega-3 PUFAs inhibit mammary gland tumorigenesis are numerous and varied. The omega-3 fatty acid-eicosanoid metabolism relationship has been the most intensely examined mechanism. Although in a particular cell line used in the studies reported herein (MDA-MB231), cell growth has been reported to be more dependent on leukotrienes than on prostaglandins (2). Nevertheless, in BHK-21 transformed cells, when inhibitors of cyclooxygenase (indomethacin) or lipoxygenase (nordihydroguaiacetic acid) were added along with the fish oil, there was no diminution in the inhibitor actions of the oil (3), suggesting that the involvement of prostaglandins or leukotrienes may be negligible in this process. At variance we have proposed the hypothesis that dietary fish oil inhibits mammary gland tumorigenesis, at least in part, by the generation and/or accumulation of secondary products of lipid peroxidation in the tumor tissue, a concept we have first described in recent publications (4-6).

These secondary products of lipid peroxidation (e.g., 4-hydroxynonenal), besides affecting a whole array of cellular biochemical and physiological functions (4), have been recently reported to suppress the expression of specific genes involved in the metabolic cascade leading to cell proliferation (7). Furthermore, fish oils have also been reported to decrease oncogene expression when compared to corn oil (8). This concept of modulating gene expression by dietary fatty acids and/or their oxidative products, is without doubt, the next logical step in nutritional tumorigenesis research. This will clarify the specific molecular mechanism(s) of action hindered in this phenomenon. This research might even help determine what specific fatty acid products can affect what specific types of cell, since the actions of fatty acid oxidation products appear to be cell type dependent (9). Also of interest are studies addressing the interaction of oxidative species with immune system components (lymphocytes, neutrophils, natural killer cells).

Other areas may also require further research. The nature of the different oxidative species formed by the oxidation of fish oil in vivo or absorbed from the diet remains to be determined. Assays with the capability to separate contributions of oxidative species generated from nonenzymatic lipid metabolism from those generated by enzymatic activity of the cellular lipoxygenase or cycloxygenase systems need to be developed. Also, more studies are needed in the area of tumor growth kinetics, specifically in relation to cell loss and cell proliferation. Preliminary results pointing at cell loss as a more important tumor growth kinetic parameter than cell proliferation need to be verified. Also of interest is the effect of different fats on

fatty acid metabolism enzymes, such as the desaturases [6-desaturase system has been reported (10) to be frequently modified in malignant cells]. Further studies in these previously mentioned areas may clarify important details of the physio-biochemical pathways that fats undertake in order to affect tumor growth. It is conceivable that an interplay of various mechanisms are operative in the suppression of mammary gland tumorigenesis by omega-3 PUFAs.

Clinical Implications

At present, fats contribute close to 40% of the total kilocalories in the average American diet. Fats are involved in numerous biochemical processes. Thus, the amount and type of dietary fat can influence normal physiological functions and also can contribute to the pathogenesis of various diseases. General consensus supports the recommendations that many Americans should consume less fat in their diet. However, less settled is the issue of the appropriate type(s) of fat. It is clear that different fats have distinct biochemical/ physiological functions in normal and/or pathological processes.

The substitution of foods with high levels of saturated fats with marine products has been recommended for health promotion and even treatment of certain diseases. Epidemiological and experimental data indicate that fish oils, which contain high levels of omega-3 PUFAs may help in the prevention and treatment of various clinical diseases (11-13), which includes breast cancer. In relation to fish oil, it is clear that the level of omega-3 PUFAs required to produce an antitumor effect is much higher than would normally be achieved from the diet alone. Nevertheless, the fish oil appears to have an anti-tumor effect similar to many conventional cytotoxic drugs with the added benefit of

no toxic short term side effects. Furthermore, the possibility of utilizing omega-3 PUFAs as an effective adjuvant therapy (thereby using less chemotherapeutic agents, thus producing less side effects without sacrificing treatment effectiveness) seems now within reach. This renewed interest in fish oil as both a preventive and a therapeutic substance for various conditions has overlooked potential detrimental side effects. These secondary effects may include: oxidation of low density lipoproteins (LDL) (14) which can be conduscent to coronary artery disease, suppression of the immune response (15) via oxidation of omega-3 fatty acids in the lymphocyte membrane and the oxidation of infant lipid emulsions and formulas (16) which can contribute to premature lung injuries and damage to the central nervous system and retina. In contrast to these detrimental effects, supplementation with fish oil has been found to be of benefit in reducing plasma triglycerides, reducing platelet aggregability (17) and possible therapeutic roles in hypertension (18), inflammatory diseases (19) and cancer (5). Although mechanisms for these actions remain uncertain, it is clear that lipid peroxidation cannot be ignored in our assessment of the role of long chain omega-3 PUFAs in normal and pathological processes.

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Appendix

Appendix

The concept of cell loss having on important role in tumor growth was introduced by Steel (Cell Tissue Kinet. 1968, 1:193-207). Fundamentally, there are two different ways of assessing cell loss:

1) indirectly - in which cell loss is estimated from the discrepancy between potential doubling time calculated from the earlier quasi-exponential growth of the tumor (in which cell loss should be minimal ≈0); and tumor doubling time calculated from the growth curve graph of the tumor.
2) directly - (although can be also considered indirectly, nevertheless we consider it the most direct way of assessment since it can obtain the values directly from the tumor in question). Entails monitoring the loss of radioactivity from the tumor after injection of a radioactive labelled compound (preferably I^{125}urd) which is incorporated into the DNA during the synthesis phase and released when tumor cells die. Tumors are measured at subsequent intervals as cells are lost, the total tumor radioactivity will decrease and the rate of this decrease once corrected for natural decay provides virtually a direct measure of cell loss rate. Cell loss is obtained from the knowledge of the volume doubling time (by periodic measures of tumor volume) and the slope of the radioactivity loss curve. The corrected curve can be equated with the rate of loss of cells from the tumors.

Many researchers have estimated cell loss by scanning techniques which require invasive methods such as excision of the tumors and prior chemical extractions of DNA of the tumor sample which is very time-consuming. In addition to being time-consuming, the tumor sample can

only be measured once. This increases the variability plus increases the number of animals and/or tumors needed for an experiment. The use of external scanning techniques seems to be a more convenient way to estimate the total radioactivity loss throughout the measuring period in a localized superficial tumor. Some advantages of employing these methods are:

1) Is a simple virtually direct measurement, that perturbs minimally the tumor in question. Also it does not rely on calculated vs. measured parameters (such as doubling times). In addition it does not require any time consuming methodology as chemical extractions, autoradiography and/or microscopic work.
2) Requires fewer animals than any invasive method that need to excise tumors for the measurement of cell loss. Also provides in vivo measurements.
3) Individual tumors are easily assessed. This individual assessment of tumors decreases variation considerably. It provides the opportunity to each tumor to serve as its own control.