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Tumor Growth Dynamics: Dietary Fish Oil Induced Inhibition of Human Breast Carcinoma Growth, a Phenomenon of Reduced Cellular DNA Synthesis or Increased Cell Loss?

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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.

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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

Table 1. Diet composition.

Ingredient1	Amount
	g/100 g diet
Fat2	20
Casein3	20.17
DL-Methionine	0.35
Dextrose	32.18
Sucrose	16.09
AIN Mineral mix4	4.13
AIN Vitamin mix4	1.18
Cellulose5	5.9

[X]A11 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 -a-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.

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.

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

Fatty acidsa	Corn oil	Menhaden oil
Caprylic (8:0)	-	-
Capric (10:0)	-	-
Laurie (12:0)	-	-
Myristic (14:0)	-	8
Palmitic (16:0)	10.1	28.9
Palmitoleic (16:1)	-	7.9
Stearic (18:0)	1.6	4
Oleic (18:1)	31.4	13.4
Linoleic (18:2)	56.3	1.1
Linolenic (18:3)	-	1
Eicosapentaenoic (20.5)	-	10.2
Docosahexaenoic (22.6)	-	12.8

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

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% 0_2: 5% $C0_2$ 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 /m 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: Bromodeoxvuridine (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 $C0_2$ 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 $5x10^{s}$ to $1xl0^{6}$ 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 µg/ml of PBS containing 10 ^g/ 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 10µg/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 fPCNA) 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 HC1). 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 1% BSA. Cells were then incubated at room temperature for 30 min. Cells were washed and resuspended in 100 µl of PBS containing 10 ng/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 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 1N HC1). 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 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(l-7)-cpm(0)] vs time (days), ln=natural log

cpm(l-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:

$Ø=(Ti/2+TD)$ Begg's formula (13)

Ø =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)

$Ti/_2$=time (days) for I^{125}Urd emission from the tumor to reach 1/2 of initial (time 0) emission rate

Ø =1-Tp Steel's Formula (12)

Td

Ø =cell loss factor=cell loss rate expressed as percent of the cell birth rate Td=tumor doubling time in days (calculated by determining the number of days for tumor to double in size)

Tp= 1n2

KL

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% C0/10% FO and 5% CO/15% FO. The animals fed 10% C0/10% FO had a higher tumor volume than those fed 5% C0/15% 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

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

Diet	Number of tumors	Mean tumor volume (cm iS.E.)	Mean H3-thymidine autoradiographs (# labeled tumors cells/mm of tissueiS.E.)e	Brdu labeling- index (% tumor cells showing Brdu uptake 1S.E.)e
1 week on diet	(Study 1)			
Corn oil	15	0.12±0.03	n.d.	20.9611.69(15)
Fish oi1b	16	0.09±0.03	n.d.	19.8511.93(16)
2 weeks on diet	(Study 2)			
Corn oila	15	0.12±0.03	138.01112.18(15)	7.2611.30(14)
Fish oi1b	9	0.07±0.02	134.70115.07(9)	10.4211.92(9)
4 weeks on diet	(Study 3)			
Corn oila	55	1.2410.11	43.5412.40(55)	n.d.
Fish oi1b	40	0.6010.073	41.6012.49(40)	n.d.
4 weeks on diet	plus diet .	supplementation (Study 4)		
Corn oila	25	1.5010.15	45.3612.98(25)	16.6512.19(25)
Fish oi1b	21	0.9110.109	43.1912.47(21)	11.6411.17(21)
Fish oil +	17	1.48l0.29f	45.4614.63(17)	13.8811.25(17)
antioxidants				
Fish oil +	10	0.2410.07h	43.8615.83(10)	n.d.
j				
iron				
6 weeks on diet	(Study 5)			
Corn oi1a	50	1.55±0.16	28.5212.04	14.1710.85(42)
i				
Fish oil	55	0.84±0.08g	25.8911.82	12.7610.80(33)
10 weeks on diet	(Study 6)			
Corn oil	8	1.20±0.34	45.79+6.29(8)	4.3111.11(6)
Fish oil	9	1.19±0.23	40.5413.80(9)	4.3010.83(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).
19% menhaden oil/1% corn oil + ferric citrate (3 g/kg diet) [e]number of tumors examined in parenthesis.
[f/g/h/]$p<0.05$.

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

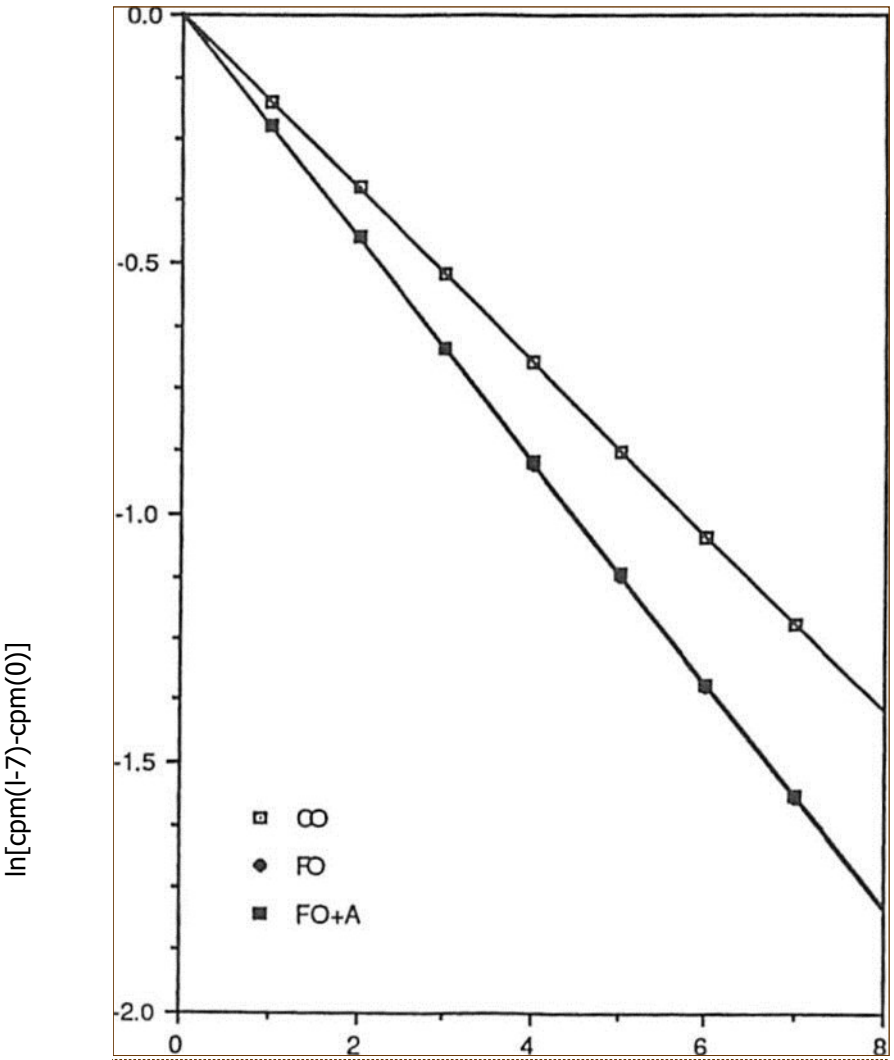

Figure 1. Rate of I^{125}Urd loss from human breast carcinomas in athymic nude mice fed corn oil (CO), fish oil (FO) and fish oil supplemented with antioxidants (FO+A) for a period of 6 weeks. Rates were 0.174±-0.01 (N=52), 0.224±0.01 (N=45) and 0.223±0.02 (N=14) for CO, FO and FO+A, respectively, $p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

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:

$$\emptyset = 1 - \frac{Tp}{Td}, \text{ where } \emptyset = \text{the cell loss factor.}$$

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 semi logarithmic plot is defined as T1/2 (time to halve the radioactivity). The modified adaptation of Steel's formula is:

$$\emptyset = Td/T½+Td)$$

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

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 tumors	of Mean tumor volume (cm 1 S.E.)	Brdu labeling- index (% tumor cells showing Brdu	PCNA labeling- index (% tumor cells PCNA positive
Corn oila	24	1.40±0.21e	4.0210.52 (24)	14.1710.85 (24)
Fish oi1b	18	0.43±0.llf	2.6310.38 (18)	12.7610.80 (18)
Fish oil +	7	1.03±0.18e	2.1410.47 (7)	17.4712.86 (7)
antioxidantsc				

[a]20% corn oil.

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

°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	Brdu labeling- index (% tumor cells showing
Corn oil 15%/ fish oila	20	1.24±0.23c	10.1111.55(16)
Corn oil 10%/ fish oila 10%	18	0.65±0.15d	7.5011.09(17)
Corn oil 5%/ fish oi1a 15%	17	0.34±0.08d	6.2111.30(9)

Menhaden oil.

[b]number of tumors examined in parenthesis. [c/d]p<0.05.

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 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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