Omega-3 Fatty Acids Suppress Growth of SW620 Human Colon Cancer Xenografts in Nude Mice

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Abstract. *Aim: The purpose of this study was to examine the influence of fish oil on growth of colon cancer in nude mice. Materials and Methods: Xenografts were initiated in mice receiving a standard diet or diets modified with corn or fish oil. After 3 weeks, mice were sacrificed, tumours were removed and processed for lipid analysis, histopathology and high resolution magic angle spinning magnetic resonance spectroscopy. Results: Diet modified with fish oil suppressed tumour growth. Xenografts from mice receiving fish oil had higher levels of omega-3 polyunsaturated fatty acids (PUFAs) with concomitant reduced levels of omega-6 PUFAs. Furthermore, these xenografts had significantly lower levels of phosphocholine. Overall the results indicated less aggressive tumour growth in mice receiving a fish oil diet.*

Colon cancer is one of the most common forms of cancer in the more developed countries. The aetiology of this type of cancer is complex and involves both genetic and environmental factors, the most important environmental factor probably being the diet. The role of dietary fat, in particular saturated fat and polyunsaturated fatty acids (PUFAs) of the omega-3 and omega-6 type, is considered to be of major importance in the pathogenesis of this type of cancer. Even though results from epidemiological data have been inconsistent in showing an association between fat intake and colon cancer risk (1-4), studies in different animal models and cell cultures studies suggest that saturated fat and omega-6 PUFAs may have a tumour promoting effect, while omega-3 PUFAs seem to protect against colon carcinogenesis (5-8). Several mechanisms have been proposed as being implicated in the action of different types of dietary fat in colon carcinogenesis. These include increased formation of secondary bile acids (9), oxidative stress (10), changes in membrane composition or function (11), alterations in eicosanoid synthesis (10, 12), signal transduction and regulation of gene expression (13).

We previously showed that two closely related colon cancer cell lines, SW480 and SW620, are strongly growth-inhibited by omega-3 PUFAs. The observed relative growth arrest in the G_2/M-phase of cell cycle was not a result of enhanced lipid peroxidation (8). However, we observed formation of docosahexaenoic acid (DHA)-enriched cytoplasmic lipid droplets, mainly in the form of triglycerides and cholesteryl esters in SW480 and SW620 cells, respectively. Moreover, decreased levels of the nuclear form of sterol regulatory element-binding protein 1 (nSREBP1) were observed, indicating a possible connection between disturbances in lipid homeostasis and cell cycle arrest (8). To evaluate our results *in vivo*, we have extended our studies to a xenograft model of SW620 cells. In this study, we wanted to examine the influence of diets enriched with either fish oil or corn oil on the growth of SW620 cells in nude mice. In addition, we wanted to determine whether these effects were related to changes in fatty acid composition of tumours, as well as metabolic changes measured by the formation of choline metabolites. The magnetic resonance (MR) detectable choline metabolites, glycerophosphocholine (GPC), phosphocholine (PC) and choline, have attracted much interest in studies of cancer tissue as their metabolism is altered in malignant cells (14, 15). PC has been suggested as a potential biomarker of an early, noninvasive therapeutic response indicator. A significant decrease in intracellular PC was found to coincide

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with cellular responses such as tumour growth delay, cell cycle arrest and different modes of cell death in human breast cancer xenografts (16, 17).

Materials and Methods

Cell culture. The human colon adenocarcinoma cell line SW620 was obtained from the American Type Culture Collection (ATCC) (Rochville, MD, USA). Cells were cultured in Leibovitz's L-15 medium (Cambrex, BioWhittaker, Walkersville, MD, USA) with L-glutamine supplemented with fetal bovine serum (FBS, 10%) and gentamicin (45 mg/l) and maintained in a humidified atmosphere of 5% CO_2: 95% air at 37˚C.

Animals, tumour implantation and diet. Xenografts were grown in female BalbC/c nu/nu athymic mice (Møllergård breeding center, Copenhagen, Denmark, n=25). The mice were aged 8-9 weeks upon arrival (weight ~19 g), and were randomly distributed into three groups (kept in 6 cages). The mice were kept in a pathogen-free environment. The room temperature was kept at 25±0.5˚C, with light cycles set to 12 h intervals, and the relative humidity was approximately 60%. After 2 weeks acclimatising, the mice were ID tagged, weighed and the three different diets were introduced (25 g per day per cage): (i) standard rodent diet (RM1(E); Special Diets Services, Scanbur AS, Nittedal, Norway), n=8; (ii) rodent diet modified with corn oil (12% calorie, AIN-93G; BIOSERV, Frenchtown, NJ, USA), n=9; (iii) rodent diet modified with fish oil (EPA/DHA 12% calorie, AIN-93G; BIOSERV), n=9. The total amount of fat was the same in diets supplemented with either corn or fish oil, while the total amount of fat was considerably less in the standard diet (Table I). Cages and water bottles were changed and autoclaved every day and once a week, respectively. The mice were given sterilised drinking water.

Three weeks (20 days) after the introduction of diet regimes, xenografts were initiated by injecting SW620 cells (1.85×10^6 cells, 100 μl) subcutaneously into the flanks of the right hind leg of each mouse. Xenograft initiation was performed under anaesthesia (Hypnorm:Dormicum:sterile water, 1:1:2, 1 ml/kg bodyweight). The cells used for injection were obtained by trypsinization of monolayer cultures, and more than 90% viability was ensured by tryphan blue exclusion assay. The tumour diameters were measured with digital callipers and tumour volume was calculated based on the measurements of two perpendicular diameters (a and b) assuming the third diameter to be equal to the shortest of the two measured. This gives the formula for the volume of a prolate ellipsoid, $V=(\pi ab^2)/6$.

The animals were sacrificed three weeks (23 days) after injection of SW620 cells. Several biopsy samples were excised from each xenograft and immediately stored on liquid nitrogen until high resolution magic angle spinning (HR MAS) MR spectroscopy and lipid extraction could commence.

MRI and histology. Anatomical MR images of the xenografts were obtained during a two-day period before mice were sacrificed (n=6, two from each group) on a 2.35T BRUKER Biospec system (BRUKER, Karlsruhe, Germany) by a multi-slice multi-echo T1-weighted sequence (MSME; BRUKER) with a repetition time of 1000 ms, total echo time of 8.8 ms, field of view of 3×3 cm, and a slice thickness of 5 mm. MRI was performed under anaesthesia Hypnorm:Dormicum:sterile water (1:1:2, 1 ml/kg bodyweight).

Table I. *Composition of the experimental diets used in this study**.

Profile	Standard	Corn oil (12% calorie**)	Fish oil (12% calorie**)
Protein	14.38%	18.5%	18.5%
Fat/oil	2.71%	5.01%	5.01%
Fiber	4.65%	4.31%	4.31%
Ash	6.00%	2.50%	2.50%
Moisture	10.00%	8.10%	7.90%
Carbohydrate	61.73%	61.58%	61.78%

*Fish oil, EPAX 3000 TG, was obtained from Pronova Biocare a.s. (Lysaker, Norway). Diet enriched with either corn or fish oil was obtained from BIO-SERV (Frenchtown, NJ, USA). Standard diet was obtained from Scanbur AS (Nittedal, Norway). **12% of the total calorie content of the diet resides from the corn/fish oil.

When groups were sacrificed, one animal from each was selected for tumour histology. The hind leg with tumour was cut off, fixed in 10% formalin and embedded in paraffin. Multiple sections were cut from each tumour and stained with haematoxylin, erythrosine and saffron.

Lipid extraction and fatty acid determination. Total lipids were extracted from tumours obtained from animals fed a fish oil-enriched diet (n=3) and corn oil-enriched diet (control) (n=3) according to a method modified after Bligh and Dyer (18). A piece of the xenografts (~0.1 g) was added to water (1 ml) and methanol-chloroform (3.75 ml; 2:1, v/v). The mixture was blended in a glass homogeniser and more methanol-chloroform-water added (4.75 ml; 2:1:0.8, v/v). The homogenate was transferred to glass-stoppered centrifuge tubes and internal standards were added (nonadecanoic acid, dinonadecanoin, trinonadecanoin and cholesteryl non-adecanoate). The mixture was thoroughly shaken and left at room temperature at 1 h with intermittent shaking. The homogenate was centrifuged (1000× g at 4˚C for 5 min) and the supernatant was further diluted with 2.5 ml of chloroform and water each and centrifuged as before. The lower chloroform layer, containing the lipids, was withdrawn and evaporated to dryness at 35˚C under a stream of nitrogen. Subsequently, the lipid residue was dissolved in chloroform (0.5 ml). The lipids were separated into free fatty acids, phospholipids, cholesteryl esters, triglycerides, cholesterol, diglycerides and monoglycerides on Bond Elut aminopropyl columns (Varian SPP., Harbor City, CA, USA) as described by Schønberg *et al*. (8).

High resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS). Tissue samples were cut (mean weight 26 mg) to fit a 4 mm o.d. rotor with inserts (total sample volume 50 μl). The samples were immersed in 40 μl phosphate-buffered saline (PBS) in D_2O in the rotor, and excess fluid was removed when assembling the rotor. Trimethylsilyl tetradeutero-propionic acid (TSP, 5 mM) was added to the buffer as chemical shift reference.

High-resolution proton MAS spectra were recorded using a BRUKER Avance DRX600 spectrometer equipped with a $^1H/^{13}C$ MAS probe (Bruker BioSpin GmbH, Germany). Samples were spun at 5 kHz and the temperature was kept at 4˚C.

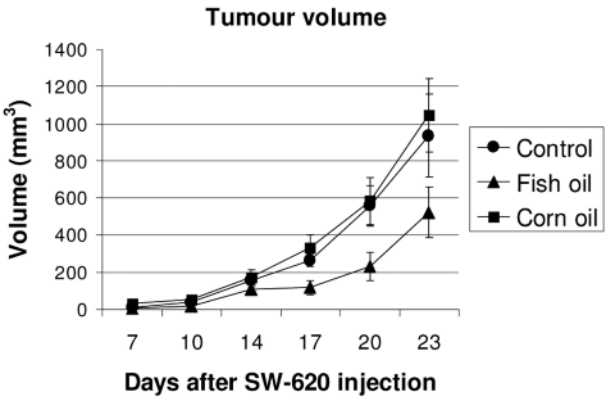

Figure 1. *Tumour volume of the human colon cancer cell lines SW620 in nude mice. Female BalbC/c nu/nu athymic mice were fed 2 weeks before injection of SW620 cells with a diet supplemented with either fish oil (▲), corn oil (■) or control diet (●). Values at each time point are the mean of all tumour-bearing animals at time. Error bars show standard error of mean (± SEM).*

Table II. *Fatty acid composition of tumours (as % of all fatty acids).*

Fatty acid	Trivial name	Corn oil (n=3)	Fish oil (n=3)
16:0	Palmitic acid	13.7	15.2
16:1	Palmitoleic acid	3.2	6.5
18:0	Stearic acid	5.5	4.4
18:1 (n-9)	Oleic acid	15.2	17.5
18:2 (omega-6)	Linoleic acid (LA)	5.2	2.2
18:3 (omega-6)	γ-Linoleic acid	<0.1	<0.1
18:3 (omega-3)	Linolenic acid (LNA)	<0.1	0.1
20:0	Eicosanoic acid	0.1	0.1
20:3 (omega-6)	Dihomo-γ-linoleic acid	0.2	0.1
20:4 (omega-6)	Arachidonic acid (AA)	3.8	1.0
20:3 (omega-3)	Eicosatrienoic acid	1.0	3.4
20:5 (omega-3)	Eicoapentaenoic acid (EPA)	<0.1	2.1
22:0	Docosanoic acid	0.1	0.1
22:4 (omega-6)	Docosatetraenoate	1.3	<0.1
22:5 (omega-3)	Docosapentaenoic acid (DPA)	0.3	1.4
22:6 (omega-3)	Docosahexaenoic acid (DHA)	2.1	3.2

Single pulse spectra were acquired with a 90˚ flip angle over a sweep width of 10,000 Hz and selective presaturation of the water (5.5 s, zgpr; BRUKER). The FID was acquired during 1.64 s and 64 transients were accumulated. A 0.3 Hz line broadening was applied before a Fourier transform into 32K points. Spin-echo experiments (cpmgpr; BRUKER) were performed using 3 s water suppression prior to a 90˚ excitation pulse. A total of 128 transients over a spectral region of 10,000 Hz were collected into 32K points, giving an acquisition time of 1.64 s. T2-filtering was obtained using a delay of 1 ms repeated 48 times, resulting in 102 ms effective echo time.

The areas of the three choline-containing compounds (GPC, PC and choline) within the spectral region of 3.20 to 3.25 ppm were determined from the single pulse acquisitions by curve fitting using a Lorenzian Gaussian function (Peak Fit; Seasolve, MA, USA). The correlation factor (r^2) was better than 0.99 for all fits. The areas were normalised to the total area below the curve (3.20-3-25 ppm) and significant differences in choline containing compounds between the three groups were determined by application of nonparametric tests.

Results

Tumour growth rates and histology. Subcutaneous injection of SW620 cells resulted in tumours in more than 90% of the animals. Two mice from the group receiving a fish oil-enriched diet did not develop tumours; one of these had to be sacrificed 10 days after initiation of xenografts due to wounds caused by other mice. However, all mice receiving standard diet or corn oil had clearly measurable tumours at this time point. Body weight data showed little difference within the two groups (data not shown). Tumour growth curves based on mean tumour volume for each group (±SEM, standard error of mean) are given in Figure 1. The tumour size was significantly smaller in animals fed a fish oil enriched diet than those fed either a diet enriched with corn oil or the standard diet on day 14, 17, 20 and 23 after injection of SW620 cells (p=0.028) (Figure 1). By day 20 after cell injection, mean tumour volume in the mice fed with the standard diet and corn oil diet approached nearly 600 mm^3, while tumours in animals fed a fish oil-enriched diet were approximately 200 mm^3. No clear difference in tumour latency was observed between control, corn oil and fish oil groups. The growth curve may also indicate that corn oil actually induced more aggressive growth, as the average volume of the xenografts in this group was higher at all time points (though not statistically significant).

Anatomical MRIs of the hind leg with xenograft from a mouse fed on a corn oil and a fish oil diet are given in Figure 2 (A and B respectively). Histological examination showed malignant tumours with considerable mitotic activity, as well as 10-15% central necrotic areas. Tumours infiltrated striated musculature, connective tissue and subcutis. Only in the tumour in the mouse fed a corn oil-enriched diet was the dermis infiltrated.

Fatty acid composition. Lipid extraction from tumours followed by analysis of different lipid fractions revealed no significant differences in the relative amount of fatty acids distributed between the different lipid classes in tumours from animals fed a fish oil-enriched diet and those fed a corn oil-enriched diet (Figure 3).

In both groups, lipids accumulated mainly as phospholipids, triglycerides and cholesteryl esters. However, when investigating the total fatty acid composition, tumours from animals fed a fish oil-enriched diet had a higher level of omega-3 PUFAs compared to tumours from animals fed

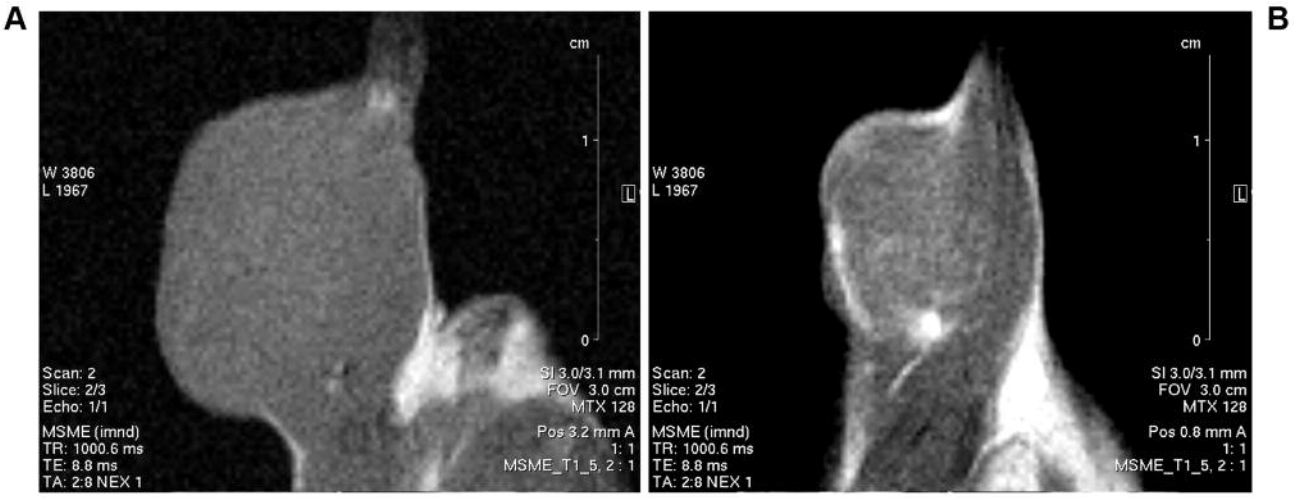

Figure 2. *Anatomical MRI (MSME, TR 1000 ms, TE 8.8 ms, FOV 3×3 cm) of the hind leg of mice with SW620 xenograft. A, Mouse fed on a corn oil-enriched diet; and B, mouse fed a fish oil-enriched diet.*

a corn oil diet, while the level of omega-6 PUFAs was substantially decreased (Table II). Table II shows an increase from <0.1 to 2.1% (EPA), 1.0 to 3.4% (eicosatrienoic acid), 0.3 to 1.4% (DPA) and 2.1 to 3.2% (DHA), and a decrease from 5.2 to 2.2% (LA), 3.8 to 1.0% (AA) and 1.3 to <0.1% (docosatetraenoate) when comparing the fatty acid profile of these tumours.

Figure 4 illustrates the differences between the total proportions of monounsaturated fatty acids (MUFAs), omega-3 and omega-6 PUFAs in tumours from animals fed a fish-oil enriched diet compared to the corn oil-fed mice. The amount of omega-6 and omega-3 PUFAs in the two groups was almost inversely correlated, indicating a partial replacement of omega-6 PUFAs by omega-3 PUFAs in the fish oil group. A higher proportion of MUFAs (16:1 and 18:1 n-9) was also observed in the fish oil group when compared with corn oil control group (Table II).

High-resolution NMR spectroscopy. Representative spin-echo proton HR MAS MR spectra of the xenografts of the major metabolites are given in Figure 5. The spectra were highly resolved. Broad resonances from lipids and macromolecules, as well as sharp peaks from smaller compounds, such as lactate, amino acids and cholines were assigned (Figure 5). Curve fitting of the choline-containing compounds demonstrated a significantly higher (p=0.011, Mann-Withney U-test) level of PC in the tumours of animals fed a diet supplemented with corn oil compared to those on a fish oil-enriched diet, thus possibly indicating less aggressive tumour growth in the fish oil group. This change in PC level is also illustrated by the expanded spectral region of interest in Figure 5.

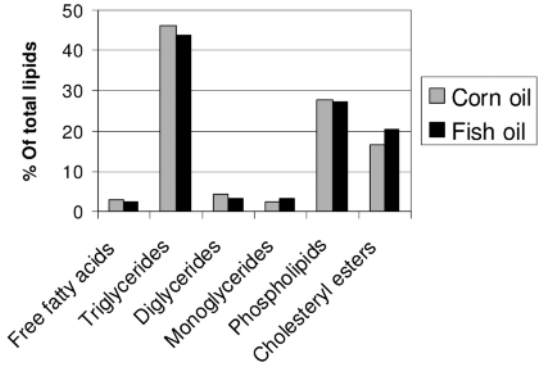

Figure 3. *Amount of lipid (% of total) in different lipid fractions in tumours from mice fed a diet either enriched with corn oil (control) or fish oil. Values represent the mean of three separate experiments.*

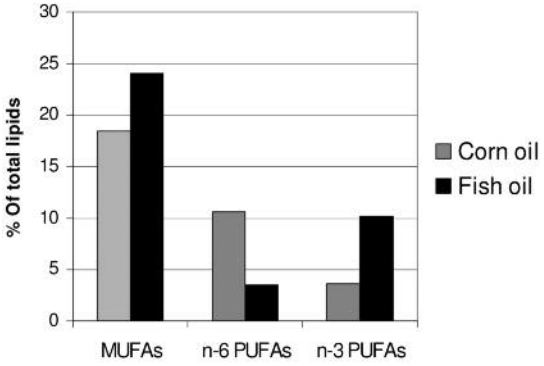

Figure 4. *Amount of lipid (% of total) accumulated as monounsaturated fatty acids (MUFAs), omega-6 and omega-3 PUFAs in tumours from mice fed a diet either enriched with corn oil (control) or fish oil. Values represent the mean of three separate experiments.*

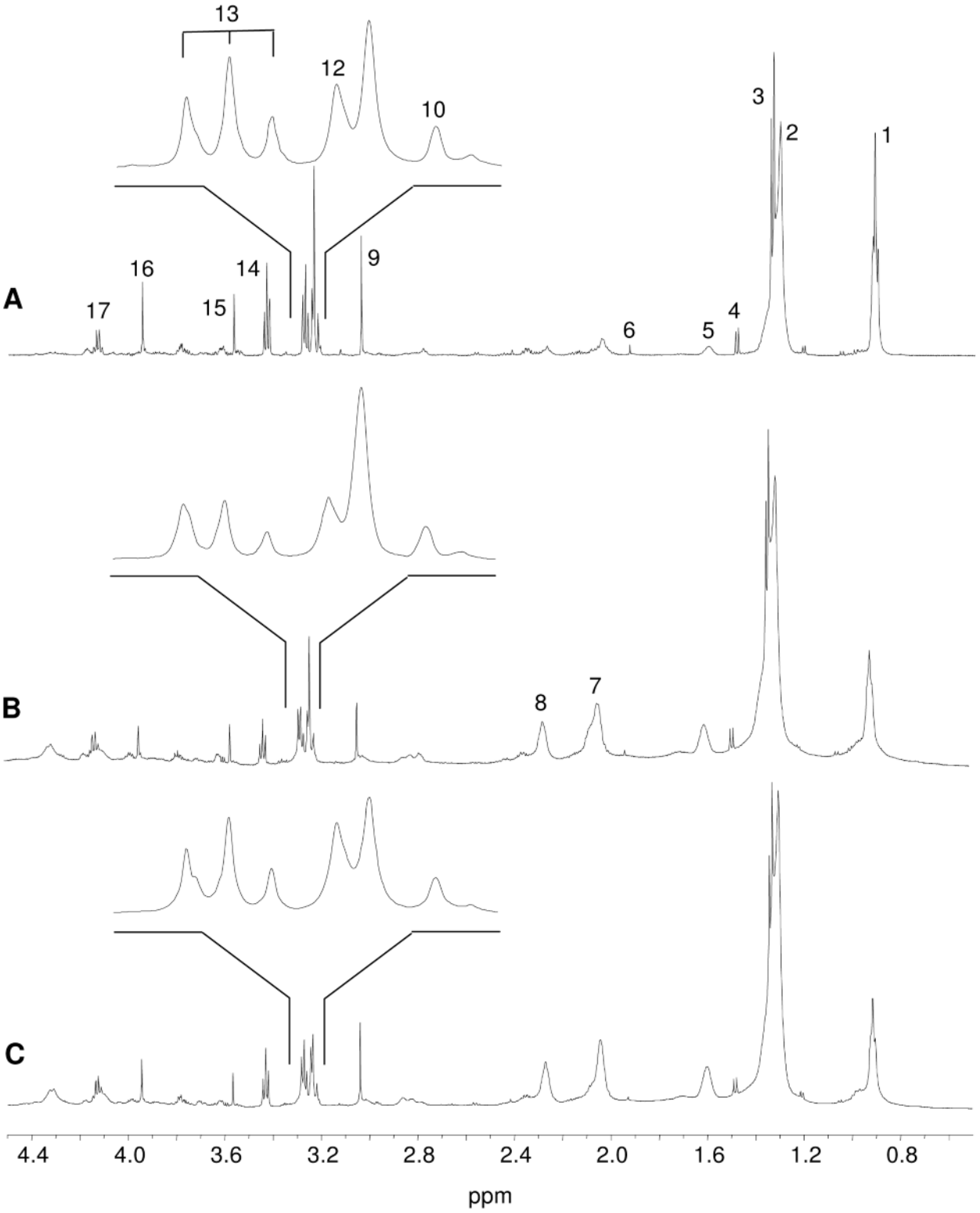

Figure 5. *Proton HR MAS MR spin-echo spectra of intact tumour tissue from mice fed standard diet (A), corn oil-enriched diet (control) (B) and fish oil-enriched diet (C). Assignments: (1) fatty acids –CH_3, (2) fatty acids (-CH_2-)$_n$, (3) lactate –CH_3, (4) alanine –CH_3, (5) fatty acids –CH_2-CH_2-CO-, (6) acetate –CH_3, (7) fatty acids –CH=CH-CH_2-CH_2-, (8) fatty acids –CH_2-CH_2-CO-, (9) creatine CH_3, (10) choline N$(CH_3)_3$, (11) phosphocholine N$(CH_3)_3$, (12) glycerophosphocholine N$(CH_3)_3$, (13) taurine N-CH_2, (14) taurine S-CH_2, (15) glycine αCH_2, (16) creatine CH_2, (17) lactate CH. Assignments are based on reference (35).*

Discussion

Previously, we showed that SW620 human colon cancer cells are growth inhibited by omega-3 fatty acids *in vitro* (8). DHA induced cell cycle arrest that, at least in part, was targeted to the G_2/M-phase and was possibly related to accumulation of cholesteryl esters (8). In the present study, we demonstrate that a fish oil-enriched diet was able to (i) suppress tumour growth of SW620 cells transplanted into athymic mice, (ii) increase the proportion of omega-3 PUFAs in tumours and (iii) decrease the level of phosphocholine in xenografts. These results show that omega-3 fatty acids possess significant tumour-suppressing properties *in vivo*, supporting our previous findings *in vitro*.

Several animal studies have convincingly shown that both the type and amount of dietary fat are important in colon carcinogenesis; a diet rich in saturated fatty acids and omega-6 fatty acids are considered tumour promoting, while diets enriched in omega-3 fatty acids may decrease risk of colon cancer development (19-21). This study indicates less aggressive tumour growth in the animals fed a fish oil-enriched diet compared to these fed with a corn oil-enriched or standard diet. This is in agreement with previous studies (22, 23).

The mechanisms behind these tumour-suppressing effects are not clear. In agreement with previous reports, we observed a reduction in the total proportion of omega-6 fatty acids in tumours from animals fed a fish oil diet and a concomitant increase in the proportion of omega-3 fatty acids (24-26). Dietary supplementation with omega-3 PUFAs leads to reduced linoleic acid desaturation and partial replacement of arachidonic acid (AA) in phospholipids in cell membranes. A higher concentration of these fatty acids compared to arachidonic acid in cellular phospholipids and lipid bodies leads to reduced production of inflammatory eicosanoids from AA (omega-6 PUFAs) and elevated production of anti-inflammatory eicsanoids from omega-3 PUFAs (27). Several studies indicate that eicosanoids from AA have a role in the pathogenesis of colon cancer as potent mediators of inflammatory and immune responses, as well as having important roles in cellular growth and differentiation (28, 29). The mechanism by which omega-3 PUFAs exert their antiproliferative effect and induce apoptosis is most likely complex. Thus, omega-3 PUFAs may inhibit colon carcinogenesis through competitive inhibition of AA-derived eicosanoids by modulation of cyclooxygenase 2 (COX-2) (30).

MR measured decrease in tumour PC has been explored as an early, noninvasive therapeutic response indicator (31, 32). A significantly higher proportion of PC was found in the xenografts from mice receiving a control diet (corn oil-enriched diet) when compared to those receiving a fish oil enriched diet. Increased concentration of PC in relation to cancer has frequently been observed. Moreover, high levels of GPC and PC and low levels of choline indicate activation of the phosphatidylcholine pathway and consequently a high membrane turnover and/or activation of cell proliferation and survival signalling. In breast cancer, up-regulation of choline transport through the activation of choline kinase in addition to increased catabolism mediated by increased phospholipase C activity, have been found to explain these observations (33, 34). In addition, PC has been found to serve as a biomarker reflecting up-regulation of specific choline transporters and choline kinase genes (14). Based on this, tumour growth seems to be less aggressive in the mice receiving a fish oil-enriched diet.

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