Inhibition of DNA replication by fish oil-treated cytoplasm is counteracted by fish oil-treated nuclear extract

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ALTHOUGH THE ASSOCIATION between dietary fat and cancer remains controversial, several hypothetical mechanisms have been proposed to explain the relationship between fat and cell proliferation (29, 32, 54, 69). Most of these mechanisms take into account the participation of lipid molecules in signal transduction pathways (24, 26, 41, 46). Arachidonic acid (AA), an essential polyunsaturated fatty acid (PUFA) of the n–6 family, is a component of cell membranes and a precursor of prostaglandin synthesis in mammalian cells (5, 7, 23, 44, 52). Both AA itself and one of its major metabolites, prostaglandin E2 (PGE2), play important roles in modulating the activity of protein kinase C and the complex signaling pathway involving mitogen-activated protein kinase (5, 7, 19–21, 23, 49, 52). Diets high in n–6 PUFAs have been generally associated with increased cell proliferation, thus positively contributing to the rate of cancer growth (50). On the other hand, diets rich in n–3 fatty acids, such as fish oil, are often considered to be inhibitory on cell proliferation due to reduction of AA in the plasma membrane and the consequent decrease in PGE2. In fact, several studies have associated fish oil with anti-cancer activity (9, 15, 20, 21, 23, 28, 59).

Evidence that fish oil inhibits cell proliferation is derived from experimental animal models as well as in vitro cell culture studies. Assuming that dietary fish oil reduces plasma membrane AA in favor of n–3 PUFA, one expects a decrease in the transition of proliferating G1 cells into S phase. This type of regulation at the G1 restriction point stems from the relationship among mitogenic signaling pathways, G1 cyclins, and the phosphorylation state of retinoblastoma protein and characterizes the typical mechanism by which extracellular factors affect cell proliferation (1, 2, 12, 33, 38, 48, 53, 56–58). However, studies in our laboratory in fish oil-treated cultured cells in conjunction with 5-bromo-2′-deoxyuridine (BrdU) pulse labeling and bivariate BrdU/NA flow cytometry suggested that the G1 to S phase transition was not affected (present study and Ref. 35). Instead, fish oil-treated cells were more typically characterized by a longer S phase duration despite a normal G1 to S phase transition. Because DNA replication in eukaryotic cells starts at multiple sites called replication origins (22, 60), we proposed that fish oil treatment interfered with the spatial and/or temporal organization of these replication origins in a manner causing S phase lengthening. In support of this hypothesis, we were able to document a spatial change in the location of the well-characterized replication origin, ori-β, in the dihydrofolate reductase...
locus in exponentially growing, fish oil-treated Chinese hamster ovary (CHO) cells (35).

To determine whether fish oil exerted its effect directly on the replicating nucleus or through signaling pathways mediated by the cytoplasm, we used a replicating cell-free system derived from HeLa cells (40). In this system, nuclei separated from synchronized HeLa cells and reconstituted with a cytosolic extract can be induced to start DNA replication (40). Therefore, if fish oil acted directly on the nucleus, we expected to observe a reduced rate of DNA replication in the nuclei derived from fish oil-treated cells, regardless of the source of the cytosolic extract. In the present study, we measured DNA replication by using BrdU pulse labeling and bivariate DNA/BrdU flow cytometry in reconstituted HeLa systems where the individual components were derived from either oil-treated or control cells. For comparison, we also analyzed cell proliferation kinetics in fish oil-treated HeLa cells by using a similar flow cytometric method. Separate controls based on growth media supplemented with either egg phosphatidylcholine or corn oil were included for both the whole cell and the reconstituted system. Our results clearly indicate that fish oil exhibited a strong inhibitory effect that was mediated through the cytosolic extract. Interestingly, fish oil-treated nuclei, but not control nuclei, were able to escape this inhibitory effect on DNA replication, suggesting that fish oil-treated nuclei can counterbalance the inhibitory effect of fish oil-treated cytosol.

MATERIALS AND METHODS

Preparation of Oil Emulsions

The preparation of oil emulsions followed mainly a procedure described by Fox and DiCorleto (27). Briefly, fish oil or corn oil (General Nutrition), respectively, was dissolved in chloroform to which 5% (wt/wt) 1-a-phosphatidylcholine from egg yolk (eggPC; Sigma) and 0.03% (wt/wt) butylated hydroxytoluene (as antioxidant) were added. The solutions were dried under a nitrogen stream, placed under vacuum overnight to remove residual solvents, and resuspended in phosphate-buffered saline (PBS) at an oil concentration of 15 mg/ml. To prepare a control solution, only eggPC and butylated hydroxytoluene were mixed and dried; PBS was added at the same ratio as for the oil emulsions.

Cell Culture and Oil Treatment

HeLaS3 cells (American Type Culture Collection) were cultured as exponentially growing monolayers on 100-mm cell culture plates in F-12K medium supplemented with 10% fetal calf serum, 108 U/ml penicillin, and 0.11 mg/ml streptomycin (all GIBCO-BRL).

Fish oil emulsion or a corresponding volume of control solution was added to exponentially growing cells at a final oil concentration of 150 µg/ml. Medium and emulsion were replaced daily, and the treatment was continued until the experiment was performed.

Experimental Part I: Cell Cycle Kinetics

Growth characteristics. HeLa cells were plated on day 0 and then treated for 5 consecutive days with control, corn oil, or fish oil emulsion. On day 5 of the treatment, the cells were harvested and then reseded at 1–2 × 10⁶ cells per plate. For the following 4 days, the treated cells were counted every 20–28 h by use of a hemacytometer and trypan blue (GIBCO-BRL). At each time point, cells were aliquoted in triplicates and each triplicate sample was counted two to three times. The growth curve for each treatment group was determined from three separate experiments. The rate of exponential cell growth (first-order rate constant \( K_d \)) was derived from semi-logarithmic growth curves by regression analysis, and the actual doubling time (\( T_d \)) was calculated from the following equation

\[
T_d = \ln 2/K_d
\]

BrdU pulse-chase labeling procedure. For kinetic analysis of the cell cycle, HeLa cells were grown at 37°C in the same manner as described for the growth curves. On day 5, cells were reseded, and treatment was continued for one more day to reestablish exponential growth. Cells were then pulse-labeled for 1 h with BrdU (Sigma) at a final concentration of 10 µM. Subsequently, cells were either harvested immediately (0 h time point) or chased for 4 h with fresh BrdU-free medium containing the corresponding concentration of control or oil emulsion. After harvesting, cells were fixed in 70% cold ethanol and stored at −20°C until further processing for flow cytometric analysis as described below.

Double-staining procedure and flow cytometry of labeled cells. Fixed cells were double-stained for DNA and BrdU content by a standard protocol as previously described (35). Briefly, fixed cells were washed with PBS and treated with 2 N HCl containing 0.5% (vol/vol) Triton X-100 (Sigma) for 1 h at room temperature to denature the DNA and improve antibody binding. Subsequently, cells were neutralized with 0.1 M sodium tetraborate (pH 8.5) and washed twice with PBS containing 0.5% (vol/vol) Tween 20 and 1% (wt/vol) bovine serum albumin (wash solution). After pelleting, cells were resuspended in 50 µl of wash solution, and then 15 µl of FITC-conjugated monoclonal anti-BrdU antibodies (PharMingen) were added and cells were incubated for 1 h in the dark. To stain for DNA content, cells were then washed with PBS and incubated in the dark for 1 h with 20 µg/ml propidium iodide (PI; Sigma) in PBS.

Afterwards, the double-stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson) at an excitation wavelength of 488 nm and a laser power of 15 mW. The red fluorescence from PI was collected through a 585-nm band-pass filter, and the green fluorescence from FITC-labeled anti-BrdU antibodies was collected through a 530-nm band-pass filter. The red fluorescence was calibrated by adjustment of the G0/G1 peak to a fixed channel number. Data from 10⁶ cells were recorded in a 1,024 × 1,024-channel distribution showing the linear amount of total DNA (red) and the logarithmic amount of BrdU (green) by using the software CellQuest (Becton Dickinson).

Kinetic analysis of flow cytometric data. To characterize the cell cycle kinetics, the recorded flow cytometric data were analyzed by using two different methods. First, we determined the distribution of cells in each phase of the cell cycle by mathematical analysis of the DNA histograms (red fluorescence only) using the computer program ModFit (Verity Software House, Topsham, ME). Second, we determined the progression through the cell cycle by analysis of the bivariate contour plots derived from the double-stained cells (red and green fluorescence; see e.g., Fig. 2) using the software program IsoContour (Verity Software House) as described below.
In DNA/BrDU contour plots (see e.g., Fig. 2), the populations of cells in G0/G1 and G2/M phase as well as the population of BrDU-labeled cells were gated and the mean red fluorescence of each population (F_L, F_G0, and F_G2, respectively) was determined. The position of BrDU-labeled cells relative to cells in G1 and G2 phase, formally defined as relative movement (RM), was calculated according to the following equation (6)

\[ RM(t) = \frac{F_L - F_G2}{F_G0 - F_G1} \]  

where RM(t) is the relative movement of BrDU-labeled cells measured at either 1 or 5 h following BrDU pulsing.

After a 4-h chase with BrDU-free medium, the BrDU-labeled cells could be separated into labeled divided (f_d) and labeled undivided (f_u) subgroups, respectively (see Fig. 2) and were quantified by gated analysis. On the basis of these fractions, a cell cycle parameter \( v \) was calculated according to the definition of White et al. (68) by using the following equation

\[ v = \ln \frac{1 + f_u}{1 - 0.5f_d} \]  

This parameter \( v \) is related to the potential doubling time \( (T_{pot}) \) and the DNA synthesis time \( (T_S) \) by the following relationship

\[ T_{pot} = \ln 2 \frac{T_S}{v} \]  

The parameter was subsequently used to calculate the cell production rate \( (c) \), according to the following equations, as previously described by White et al. (67)

\[ c = \left[ v - 2 \cos \left( \frac{\theta - 2\pi}{3} \right) - 1 \right] / t \]  

where

\[ \theta = \arccos \left( 1 - \frac{3v + f_u(t)[1 - RM(t)]}{1 + f_u(t)} \right) \]  

and \( t \) is the time interval between BrDU labeling and flow cytometric analysis. For \( c \) was estimated, \( T_S, T_{pot} \), and the time spent in G2/M phase \( (T_{G2+M}) \) were derived from the following relationships (67)

\[ T_S = v/c \]  

\[ T_{pot} = \frac{\ln 2}{c} \]  

\[ T_{G2+M} = t + \frac{\ln [1 - f_u(t)/2]}{c} \]  

Finally, an estimate of cell loss was derived from comparison of \( T_{pot} \) and \( T_A \) according to the following equation for the cell loss factor \( (\Phi) \), expressed as a fraction (62)

\[ \Phi = 1 - \left( T_{pot}/T_A \right) \]  

Experimental Part II: Cell-Free Reconstitution Experiments

Synchronization in S phase. In preparation of a reconstitution experiment, culture media were supplemented with an oil or control emulsion for 4 days. Cells were reseeded on day 5, and the treatment with either emulsion was continued. On day 6, exponentially growing cells were synchronized in S phase by a single thymidine block for 24 h. A final concentration of 2.5 mM thymidine (Sigma) was found to be sufficient for synchronization of HeLa cells in early S phase. Subsequently, cells were harvested and processed as described below.

The adequacy of the synchronization procedure was verified by flow cytometry. Briefly, fixed synchronized cells were washed with PBS, incubated with 0.5 mg/ml RNase A in PBS for 2 h at room temperature, washed with PBS, and subsequently incubated with 20 μg/ml PI in PBS for several hours or overnight. Flow cytometric analysis of DNA histograms was performed as explained for Experimental Part I.

Preparation of nuclei and cytosolic extracts. A procedure described by Krude and colleagues (39, 40) was followed with minor modifications. Briefly, cell monolayers were washed once with ice-cold Hanks’ balanced salt solution (HBSS). After further addition of 2 ml of ice-cold HBSS, cells were scraped off the substratum at 4°C and centrifuged at 1,000 g for 10 min in an MR18.22 centrifuge (Jouan). The pellet was resuspended and swollen in hypotonic buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, and 2 mM DTT) for 15 min on ice. Cells were disrupted with 1 ml of 10% NP-40 in a Dounce homogenizer (pestle B). Nuclei were checked for the presence of undisrupted cells by using trypan blue. Accordingly, the homogenization was either continued or terminated. After centrifugation for 5 min at 1,800 g, the pellet (nuclei) was separated from the supernatant (cytosolic fraction), washed with PBS, and counted with a hemacytometer.

About 1.5 × 10⁶ nuclei per Eppendorf tube were spun down and resuspended in 50–100 μl of storage solution [250 mM sucrose, 75 mM NaCl, 0.5 mM spermine tetrachloride, 0.15 mM spermidine trichloride (both Sigma), and 3% (wt/vol) bovine serum albumin] and stored at −80°C. The cytosolic fraction was kept on ice until further centrifugation at 20,000 g for 30 min. The protein concentration ranged between 3 and 12 mg/ml. Subsequently, the supernatant was stored at −80°C.

Preparation of nuclear extracts. Nuclear extracts were prepared freshly before use from isolated nuclei as reported by Krude et al. (40). Pelleted nuclei were resuspended at a concentration of 150 × 10⁶ nuclei/ml in hypotonic buffer containing a final concentration of 0.4 M NaCl. After extraction for about 2 h on ice and vortexing every 30 min, the suspension was centrifuged for 30 min at 16,000 g at 4°C. The supernatant (nuclear protein concentration of 7–11 mg/ml) was immediately separated from the pellet and kept on ice.

Reconstitution experiments. About 1.5 × 10⁶ nuclei synchronized in early S phase were used per reconstitution experiment. Nuclei in storage solution were pelletized and resuspended on ice in a reaction mixture composed of 5× reaction buffer and 5 phase cytosolic extract (containing 200–300 μg of protein per 1–1.5 × 10⁶ nuclei). The amount of reaction buffer added was adjusted so that the total volume of the reaction solution yielded a final concentration of 40 mM HEPES, pH 7.5, 7 mM MgCl₂, 3 mM ATP, 0.1 mM each of GTP, UTP, and CTP, 0.1 mM each of dATP, dGTP, and dCTP, 0.5 mM DTT, 40 mM creatine phosphate, 5 μg of phosphocreatine kinase (both Boehringer-Mannheim), and either 0.1 mM BrDU triphosphate (BrdUTP; Sigma) or dTTP. The reconstitution reaction was started by transferring the samples from ice to 37°C. The nuclei were pulse-labeled for 30 min with reaction buffer containing BrdUTP. Subsequently, nuclei were either fixed immediately (0 h time point) or spun down, resuspended in fresh reaction buffer containing dTTP, chased for 2 h at 37°C, and then fixed (2 h time point). In all reconstitution experiments, the chase time was limited to 2 h because nuclear integrity became visibly compromised afterwards.
In reconstitution experiments with nuclear extracts, 100–130 μg of nuclear protein extract, 180–250 μg of cytosolic extract (both from cells treated with fish oil), and reaction buffer were added to 1.5 × 10⁶ nuclei from control cells, and nuclei were then BrdU-UTP-labeled and chased as explained above. Nuclei were stained with PI and FITC-conjugated anti-BrdU antibodies and analyzed by flow cytometry in the same manner as the cells.

Statistical Analysis

Statistical comparison of multiple groups was evaluated by one-way analysis of variance (ANOVA). Subsequently, specific comparisons between two means were made by the Student’s t-test only when ANOVA showed significant overall difference (P < 0.05). Comparison of data derived from two separate means, such as ΔRM, was made by a modified Student’s t-test, known as “Welch’s approximate t” as described by Zar (70). All statistical analyses were performed by using the software programs Excel and SigmaPlot.

RESULTS

Fish Oil Treatment Decreases the Proliferation Rate of HeLa Cells

To ensure exponential growth conditions, we determined the growth curves for all three treatment groups. After 5-day treatments with eggPC, corn oil, and fish oil, cells were reseeded and grown for four consecutive days in control or oil-containing medium. Daily aliquots were used to determine mean cell numbers, as summarized in a semilogarithmic plot in Fig.1. Whereas control and corn oil-treated cells showed very similar growth rate constants (Kg, slope), fish oil-treated cells exhibited a smaller value of Kg. The actual doubling times (Td) were determined from the slope of each straight line according to Eq. 1, and the values are given in Table 1. Fish oil-treated cells exhibited a significant increase in Td (29.3 ± 1.5 h) by ~30% compared with control (22.1 ± 0.3 h) or corn oil-treated cells (22.8 ± 1.8 h), respectively (P ≤ 0.05 for fish oil vs. control and corn oil).

Table 2. Cell cycle phase distribution derived from DNA histograms

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Go/G1</th>
<th>S</th>
<th>G2/M</th>
<th>Labeling Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55.2 ± 1.6</td>
<td>25.7 ± 1.2</td>
<td>19.1 ± 0.5</td>
<td>29.0 ± 1.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>55.4 ± 1.0</td>
<td>25.9 ± 0.6</td>
<td>18.7 ± 0.5</td>
<td>26.6 ± 0.7</td>
</tr>
<tr>
<td>Fish oil</td>
<td>55.3 ± 0.7</td>
<td>27.2 ± 0.5</td>
<td>17.5 ± 0.3</td>
<td>27.5 ± 1.0</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 4 samples. Cell cycle phase distributions are percentages. Labeling index values were obtained from bivariate DNA/BrdU profiles.

Fish Oil Treatment of HeLa Cells Increases the S Phase Duration Time Without Affecting G1/S Phase Transition

First, DNA histograms were analyzed to determine the effect of oil treatment on the distribution of cells within the cell cycle. Results of the percentages of cells in each cell cycle phase are summarized in Table 2, showing no significant effects for either oil treatment on cell cycle distribution. Table 2 also shows that the labeling index, expressed as the percentage of BrdU uptake in cells after 1 h of labeling time, was also similar among the three treatment groups. However, significant differences were noted in cell cycle kinetic parameters derived from BrdU labeling and bivariate flow cytometry, as explained in MATERIALS AND METHODS. Cell cycle data were obtained by analyzing contour plots (Fig.2) from fish oil- and corn oil-treated cells recorded at 1 h (A and C) and 4 h (B and D), respectively, after BrdU treatment. As noted in Fig. 2, the population of BrdU-labeled cells can be divided into labeled undivided (fUD) and labeled divided fractions (fD). These results are summarized in Table 3. Whereas control and corn oil-treated cells show 2.8 ± 0.2 and 3.1 ± 0.3% of cells, respectively, in the fD fraction, fish oil-treated cells have a significantly lower

Table 1. Estimates of cell cycle time, actual doubling time, potential doubling time, and cell loss

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tg, h</th>
<th>Ts, h</th>
<th>Tg2-M, h</th>
<th>Tpot, h</th>
<th>Φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.1 ± 0.3</td>
<td>7.29 ± 0.32</td>
<td>4.64 ± 0.02</td>
<td>18.78 ± 1.28</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Corn oil</td>
<td>22.8 ± 1.8</td>
<td>6.90 ± 0.39</td>
<td>4.61 ± 0.05</td>
<td>19.98 ± 0.82</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Fish oil</td>
<td>29.3 ± 1.5†</td>
<td>9.88 ± 0.25†</td>
<td>4.56 ± 0.04</td>
<td>27.66 ± 1.21†</td>
<td>0.08 ± 0.004</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 5 samples. Tg, actual doubling time (Eq. 1); Ts, DNA synthesis time (Eq. 7); Tg2-M, time spent in G2/M phase (Eq. 9); Tpot, potential doubling time (Eqs. 4 and 8); Φ, cell loss factor, fraction (Eq. 10). †P ≤ 0.0001 compared with corn oil and control.
percentage of cells in this fraction (1.1 ± 0.2%) (P < 0.0005 for fish oil vs. control and corn oil). However, after 4 h of chase time, the number of cells in the \( f_{\text{iu}} \) fraction had not changed significantly for all three treatment groups. On the basis of these results, the parameter \( v \) (Eq. 3), which represents the fraction of cells traversing the S phase during one cell cycle, did not vary significantly between the corn oil and fish oil treatment groups. These findings imply equal rates of G1 to S phase transition in HeLa cells treated with fish oil, corn oil, or eggPC.

Estimates of the DNA synthesis time (\( T_S \)) were derived from the relative movement (RM) of BrdU-labeled cells and the parameter \( v \) according to the method of White et al. (67). As noted in Table 3 and Fig. 3, RM was similar in the treatment groups 1 h after BrdU pulse labeling. However, cells analyzed 4 h after BrdU labeling showed significantly lower RM values in the fish oil-treated group compared with the other two groups (\( P < 0.00005 \) for fish oil vs. control and corn oil; Table 3 and Fig. 3). Based on these values and Eqs. 5–7, the mean DNA synthesis time was determined as 9.88 ± 0.25 h in fish oil-treated cells, 7.29 ± 0.32 h in control cells, and 6.90 ± 0.39 h in corn oil-treated cells, respectively (\( P < 10^{-5} \) by ANOVA). Similarly, estimates of the potential doubling times (\( T_{\text{pot}} \)) were significantly longer in the fish oil-treated cells, whereas estimates of the time spent in G2/M phase were remarkably similar in all treatment groups (Table 1). Finally, estimates of cell loss, also summarized in Table 3.

Table 3. Kinetic parameters derived from bivariate BrdU/DNA profiles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Labeling Time, h</th>
<th>( f_{\text{iu}} )</th>
<th>( f_{\text{id}} )</th>
<th>( v )</th>
<th>RM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>29.0 ± 1.0</td>
<td>0.255 ± 0.008</td>
<td>0.635 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>1</td>
<td>26.8 ± 0.7</td>
<td>0.236 ± 0.005</td>
<td>0.623 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>1</td>
<td>27.5 ± 1.0</td>
<td>0.243 ± 0.008</td>
<td>0.632 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>26.0 ± 0.5</td>
<td>2.8 ± 0.2</td>
<td>0.245 ± 0.004</td>
<td>0.963 ± 0.010</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>25.2 ± 1.0</td>
<td>3.1 ± 0.3</td>
<td>0.240 ± 0.008</td>
<td>0.956 ± 0.006</td>
</tr>
<tr>
<td>Fish oil</td>
<td>5</td>
<td>27.5 ± 0.7</td>
<td>1.1 ± 0.2*</td>
<td>0.248 ± 0.005</td>
<td>0.865 ± 0.007*</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 5 samples. Labeling time includes BrdU pulse time (1 h) and subsequent chase time (4 h). \( f_{\text{iu}} \), fraction of labeled undivided cells (as a percentage of total cells); \( f_{\text{id}} \), fraction of labeled divided cells (as a percentage of total cells); \( v \), dimensionless function (Eq. 3); RM, relative movement (Eq. 2). *\( P < 0.0005 \) compared with corn oil and control at 5 h time point. †\( P < 0.00005 \) compared with corn oil and control at 5 h time point.
Table 1, were not significantly different among the three treatment groups. These findings implicate a slower S phase progression as the main cause of decreased cell proliferation rate in fish oil-treated HeLa cells.

**Cytoplasm From Fish Oil-Treated Cells Inhibits DNA Synthesis in Nuclei From Control Cells But Not in Nuclei From Fish Oil-Treated Cells**

To differentiate between cytosolic and/or nuclear sites of action for fish oil on DNA replication, we performed a set of reconstitution experiments using a cell-free system prepared from control, corn oil-treated, and fish oil-treated cells. In this system, S phase-synchronized HeLa cells (see e.g., Fig. 4) were used to prepare nuclei and cytosolic extracts as described in MATERIALS AND METHODS. In all, seven cell-free reconstitution systems were included in this study, as summarized in Tables 4 and 5. As expected, ~90% of the nuclei were in S phase, with a small percentage in G0/G1 and G2/M phase as depicted in Fig. 5. Thus it was possible to determine the RM of BrdU-labeled nuclei in the reconstituted system. Typical bivariate plots of reconstituted double-stained nuclei are shown in Fig. 5, A (0 h) and B (2 h). Because of the density of nuclei in the early part of S phase, it was not possible to discern movement of nuclei along the DNA axis after 2 h by simple visual inspection. However, actual movement is more readily seen in the relative distribution of nuclei along the DNA axis, as demonstrated in Fig. 5C, which is shown for illustrative purposes. In Fig. 5, A and B, the DNA axes were divided into six discrete S phase compartments in units of RM (<0.35, 0.35–0.50, 0.50–0.65, 0.65–0.80, 0.80–0.95, 0.95–1.0), and the percentage of nuclei in each compartment was determined. As expected, the majority of nuclei synchronized in S phase were found in the compartment with RM < 0.35 at 0 h. Two hours after reconstitution, the relative number of nuclei with RM < 0.35 diminished from 64.5 ± 1.6% to 56.6 ± 1.0% (P < 0.001), whereas the percentage of nuclei in the compartment of 0.35 < RM < 0.50 significantly increased from 26.1 ± 1.7% to 34.0 ± 1.9% (P < 0.005). Similarly, there were significant increases in the relative number of cells in higher RM compartments after 2 h of reconstitution (Fig. 5C). These results, showing a redistribution of BrdU-labeled nuclei from lower to higher RM compartments, indicate a significant and measurable increase in DNA content in the reconstituted nuclei.

The relative rate of DNA synthesis in a reconstituted system can be assessed from the change of RM over the 2-h time period. Values of RM for the total S phase population at each reconstitution time point are summarized in Table 4. Relative movement for reconstituted fish oil-treated systems

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclei</th>
<th>Cytosol</th>
<th>RM (0 h)</th>
<th>RM (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Control</td>
<td>0.308 ± 0.004</td>
<td>0.362 ± 0.006</td>
</tr>
<tr>
<td>2</td>
<td>Fish oil</td>
<td>Fish oil</td>
<td>0.312 ± 0.005</td>
<td>0.356 ± 0.006</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>Fish oil</td>
<td>0.308 ± 0.003</td>
<td>0.321 ± 0.002</td>
</tr>
<tr>
<td>4</td>
<td>Fish oil</td>
<td>Control</td>
<td>0.318 ± 0.004</td>
<td>0.384 ± 0.005</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 6–12 samples. Corresponding changes in relative movement (ΔRM) are shown in Fig. 6A. RM (0 h) values show no statistically significant differences as tested by ANOVA; RM (2 h) values differ significantly as tested by ANOVA analysis (P < 3 × 10⁻⁸).
Table 5. Relative movement for reconstituted corn oil-treated systems

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclei</th>
<th>Cytosol</th>
<th>RM (0 h)</th>
<th>RM (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Control</td>
<td>0.308 ± 0.004</td>
<td>0.362 ± 0.006</td>
</tr>
<tr>
<td>5</td>
<td>Corn oil</td>
<td>Corn oil</td>
<td>0.258 ± 0.006</td>
<td>0.324 ± 0.010</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>Corn oil</td>
<td>0.284 ± 0.009</td>
<td>0.354 ± 0.009</td>
</tr>
<tr>
<td>7</td>
<td>Corn oil</td>
<td>Control</td>
<td>0.275 ± 0.006</td>
<td>0.362 ± 0.006</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 6–9 samples. Corresponding ΔRM are shown in Fig. 6B. All RM values differ significantly as tested by ANOVA analysis (P < 0.005).

were noted in RM measured at 2 h (P < 10⁻⁵; by ANOVA) with the reconstitution group of fish oil-treated cytosol/control nuclei (group 3) having the smallest value (P < 0.01 for group 3 vs. groups 1, 2, or 4; by t-test). A summary of the relative rates of DNA synthesis in each of the treatment groups, expressed by the 2-h increment in RM (ΔRM), is presented graphically in Fig. 6. As noted in Fig. 6, fish oil-treated cytosol appears to reduce the relative rate of DNA synthesis by ~80% in control (untreated) nuclei (P < 0.01 for group 3 vs. group 1). In contrast, when fish oil-treated nuclei were reconstituted with fish oil-treated cytosol (group 2), no such inhibition in DNA synthesis was noted. Similarly, there was no inhibition of DNA synthesis in the fish oil-treated nuclei reconstituted with control (untreated) cytosol.

Fig. 5. Contour plots of bivariate BrdU/DNA distribution were obtained from flow cytometric analysis of nuclei isolated from control cells synchronized in early S phase and reconstituted with cytosol from control cells (group 1) (see Table 4 and Fig. 6). The nuclei were pulse-labeled for 30 min with 100 μM BrdU triphosphate (BrdUTP) and either immediately fixed (A) or chased for 2 h with dTTP (B). The notation of both axes is the same as in Fig. 2. The positions of nuclei in G0/G1, S, and G2/M phase, respectively, are indicated. C: for illustrative purposes and to give evidence of relative movement (RM) among the S phase nuclei, the RM values were grouped into 6 compartments (<0.35, 0.35–0.5, 0.5–0.65, 0.65–0.8, 0.8–0.95, 0.95–1.0). For each compartment the percentage of BrdU-labeled nuclei was determined at 0 and 2 h. *Statistical significant difference between both time points.
In the corn oil-treated groups (Table 5), differences were noted in RM at 0 h ($P < 0.0001$; by ANOVA) as well as at 2 h ($P < 0.005$; by ANOVA). However, comparison of $\Delta RM$ among the reconstitution systems of the corn oil treatment group (Fig. 6B) showed no significant differences except for group 7 ($\Delta RM = 0.087 \pm 0.008$) compared with group 1 ($\Delta RM = 0.054 \pm 0.007$) ($P < 0.01$). Thus nuclei from corn oil-treated cells appear to progress faster through S phase than nuclei from control cells when both are incubated with control cytosol.

**Nuclear Extract From Fish Oil-Treated Cells Reverses the Inhibitory Effect of Fish Oil-Treated Cytosol**

To further establish the enhancing effect of nuclear components on the DNA synthesis in the fish oil reconstitution system, we prepared nuclear extract from fish oil-treated cells and incubated the nuclei derived from control cells with fish oil-treated cytosol and fish oil-treated nuclear extract (group 8). Results of RM at 0 and 2 h as well as $\Delta RM$ are presented in Table 6 and Fig. 6C, with pertinent comparison groups. The addition of nuclear extract reversed the inhibitory effect observed with fish oil-treated cytosol and control nuclei (group 3), leading to an increase in $\Delta RM$ from 0.013 $\pm$ 0.004 for group 3 to 0.099 $\pm$ 0.023 for group 8 ($P < 0.001$). We also note that $\Delta RM$ for group 8 was larger than that for group 2, where fish oil-treated nuclei were incubated with fish oil-treated cytosol.

**DISCUSSION**

This study was conducted to further clarify the mechanism(s) by which fish oil alters the proliferation of mamalian cells. The relevance of this inhibitory effect of fish oil for the relationship between diet and cancer has been addressed in the literature. In fact, both fish oil supplementation and pharmacological treatment aiming at the inhibition of cyclooxygenase activity to alter the prostaglandin composition of cells have been proposed in cancer prevention (13, 17, 44, 51, 54). For the most part, fish oils are thought to induce changes in cell function (3, 16, 23, 27, 28, 36, 45, 47, 55, 61, 65) by replacing membrane n–6 fatty acids, such as AA, with PUFAs of the n–3 family. Consequently, multiple changes in plasma membrane function, such as transmission of receptor-induced extracellular signals to the interior of the cell, have been hypothesized (4, 14, 19, 23, 31, 42, 63–65).

**Table 6. Relative movement for reconstituted fish oil-treated systems including nuclear extract**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclei</th>
<th>Cytosol</th>
<th>Nuclear Extract</th>
<th>RM (0 h)</th>
<th>RM (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control</td>
<td>Fish oil</td>
<td>None</td>
<td>0.308 ± 0.003</td>
<td>0.321 ± 0.002</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>Fish oil</td>
<td>Fish oil</td>
<td>0.271 ± 0.006</td>
<td>0.370 ± 0.022</td>
</tr>
<tr>
<td>2</td>
<td>Fish oil</td>
<td>Fish oil</td>
<td>None</td>
<td>0.312 ± 0.005</td>
<td>0.356 ± 0.006</td>
</tr>
</tbody>
</table>

All data are given as means ± SE of 5–9 samples. Corresponding $\Delta RM$ are shown in Fig. 6C.

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We have shown previously that addition of fish oil to the diet of tumor-bearing rats reduces the growth rate of tumors and causes a prolongation of the time required for DNA replication without affecting the transition of proliferating cells from G₁ to S phase (36). These findings suggested that fish oil might interact with the DNA replication machinery at the level of the replicating nucleus separately from its putative down-regulatory effect on classic mitogen-induced signaling pathways, as had been proposed (10, 33, 53). In support of this hypothesis, we recently demonstrated a change in the spatial localization of the well-characterized ori-β replication origin in CHO cells (35).

Our main objective in the present study was to determine whether fish oil alters DNA replication by a direct effect on the nucleus and whether cyttoplasmic signaling is also involved in this inhibitory effect. For this purpose we used a cell-free DNA replicating system derived from HeLa cells that was first described by Krude et al. (40). The system allows reconstitution of control or fish oil-treated cytoplasm with either control or fish oil-treated nuclei in a crossover design. As shown by Krude et al. (40), synchronized G₁ nuclei separated from HeLa cells can be induced to assemble the DNA replication machinery if incubated in the presence of a cytosolic extract, a nuclear extract from S phase cells, and an energy-generating system. These reconstituted cell components engage in DNA synthesis for several hours. In the present study, we used S phase nuclei that had already assembled the DNA replication machinery, thus obviating the need for separate nuclear extracts in the reconstitution system. Before cell fractionation, these nuclei were exposed to fish oil in the growth medium for 5 days, a period sufficient to induce changes in S phase progression, as noted in MATERIALS AND METHODS. Therefore, if fish oil affected the assembly of replication origins by a direct effect on nuclear organization, we expected to detect differences in S phase progression in the reconstituted system composed of fish oil-treated nuclei and cytosolic extract from control cells. Similarly, the experimental design allowed testing of the possibility that fish oil mediates its effects through the cytoplasm if DNA replication is affected in the system reconstituted from fish oil-treated cytosol and control nuclei.

In the absence of prior information about the effect of fish oil on proliferation kinetics of HeLa cells, fish oil treatment of whole HeLa cells was necessary in the present study. This set of experiments was performed under conditions of exponential growth, in line with our previous characterization of the effect of fish oil in other cell lines (35). As reported here, initial studies utilizing whole HeLa cells depict a similar response to fish oil supplementation as previously noted in CHO cells (35). After 5 days of treatment with fish oil-containing medium, HeLa cells grow at a significantly slower exponential rate. This difference in actual cell proliferation rate is not explained by an increase in cell death, because flow cytometric data did not reveal evidence of apoptosis or cell fragmentation as in our previous studies with individual n-3 fatty acids (11). It also is not explained by a reduction in the entry of proliferating cells into S phase, as depicted by the kinetic parameter $v$. Only the estimate of DNA replication time, derived from the rate of progression of BrdU-labeled cells relative to the position of cells in G₀/G₁ and G₂/M phase, was significantly increased in fish oil-treated cells (Table 1). As noted in Table 1, the increase in $T_{pot}$ is predominantly explained by a longer S phase duration in fish oil-treated cells.

In the reconstituted system, DNA synthesis takes place within 2 h, as noted in the increase in relative movement and in redistribution of BrdU-labeled nuclei toward higher DNA content (Fig. 5). However, the stability of this system becomes compromised after 2 h, thus making it impossible to determine the kinetics of DNA synthesis in the later stages of S phase. For this reason, it was not possible to obtain estimates of $T_S$, $T_d$, or other cell cycle kinetic parameters from the reconstituted system. Despite this limitation in the cell-free HeLa system used in the present study, our results clearly demonstrate an inhibitory effect on DNA replication mediated by the fish oil-treated cytoplasm. Our findings also suggest that this cytosolic inhibitory signal on DNA replication caused by fish oil treatment is counterregulated at the level of the nucleus. Interestingly, nuclear extracts prepared from fish oil-treated HeLa cells can completely restore DNA synthesis in the inhibited reconstitution system. Therefore, escape from DNA synthesis inhibition and completion of the S phase in the 5-day fish oil-treated HeLa nuclei is not achieved by an alteration in nuclear structure or chromatin organization but, rather, by a soluble nuclear factor.

Although the effects of fish oil on cell proliferation are likely to be related to its content of n-3 fatty acids, this conclusion cannot be ascertained from the current study. We have included an experimental group treated with corn oil to evaluate for the effect of n-6 PUFA. Assuming that n-3 fatty acids ultimately account for the inhibitory effect, one may expect an opposite stimulatory effect if n-6 fatty acids are introduced into the system. The results of the current study are consistent with this possibility, because corn oil-treated nuclei appear to synthesize DNA at a faster rate than control nuclei. However, further studies to determine the significance of potential changes in cytoplasmic signaling and nuclear response, in relationship to n-3 and n-6 fatty acid cellular composition, are needed.

The mechanisms of DNA replication inhibition and those of the apparent recovery of the fish oil-treated nucleus in this study are only speculative at this time. DNA replication initiates at specific chromosomal sites in a complex process that remains incompletely understood in eukaryotic cells (18, 22, 25, 34, 66). The fact that the nuclei used in this study were already in the early S phase at the time of reconstitution implies that the inhibitory effect of fish oil occurred during DNA elongation. Individual steps that could potentially contribute to the total effect of fish oil include strand separation by helicase and priming, as well as actual
polymerase activity. It is also possible that the assembly of replication initiation complexes at late replication origins, which are activated in later stages of the DNA replication process, does occur in early S phase and that this may be specifically prevented by the fish oil-treated cytosolic extract. Alternatively, slowing of DNA replication in fish oil-treated cells may result from activation of the regulatory intra-S phase checkpoint system (8, 30, 37, 43). However, it is important to note that this system normally responds to DNA damage, for which we have no evidence in the current study. Furthermore, this mechanism does not explain the apparently normal DNA synthesis response in the fish oil-treated nuclei reconstituted with fish oil-treated cytosol. Obviously, further research is needed to clarify these numerous possibilities.

In conclusion, we present here evidence that fish oil treatment of proliferating mammalian cells triggers an inhibitory signal for DNA synthesis in the cytoplasm. It also appears that counterregulatory mechanisms can occur within the fish oil-treated nucleus to overcompensate this inhibition in the whole cell. Further investigations of the components of this system will be helpful in understanding the interaction among fatty acids, fats, and cell proliferation.

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REFERENCES


