Differential sensitivity of cancer cells to docosahexaenoic acid–induced cytotoxicity: The potential importance of down-regulation of superoxide dismutase 1 expression

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Abstract

Docosahexaenoic acid (DHA, 22:6 n-3), a polyunsaturated fatty acid found in fish oil, exerts cytotoxic effects on cancer cells. Although DHA was toxic toward five human cancer cell lines (MCF-7, MDA-MB-231, SiHa, Raji, and DHL-4), the lines were not uniformly sensitive. DHL-4, a bcl-2 overexpressing lymphoid line, was the most sensitive (IC50, 5.2 μmol/L) and the cervical cancer cell line, SiHa, was the most resistant (IC50, >300 μmol/L). Lipid peroxidation has been cited by others as an important component of DHA toxicity, and we confirmed that vitamin E prevents the cytotoxic effects of DHA. Lipid peroxidation was greater following DHA treatment of the sensitive DHL-4 cells than in the resistant SiHa cells, as assessed by thiobarbituric acid reactive substance generation. DHL-4 cells treated with DHA for 20 hours showed a 3.5-fold increase in thiobarbituric acid reactive substances, whereas SiHa cells showed no increase. Reverse transcription-PCR analysis detected a down-regulation of the expression of the major antioxidant enzyme, superoxide dismutase (SOD) 1, in DHL-4 cells but not in SiHa cells after DHA treatment. Knockdown of SOD1 expression in SiHa cells with small interfering RNA significantly enhanced lipid peroxidation and cytotoxicity on exposure to DHA. These results show that DHL-4 cells are highly sensitive to the cytotoxic effect of DHA and that regulation of SOD1 expression may play an important role in determining the sensitivity of different tumor cells to the cytotoxic effects of DHA. [Mol Cancer Ther 2004;3(9):1109–17]

Introduction

n-3 Polyunsaturated fatty acids (n-3 PUFA) have been recognized to have anticancer properties for over 20 years.

The effects of n-3 PUFAs on cancer have been studied by several laboratories (reviewed in refs. 1, 2), stimulated in part by studies of Eskimos, who consume PUFA-rich diets and exhibit lower rates of certain types of cancer (3, 4). Subsequent studies in cell culture and animal models showed that n-3 PUFAs, such as docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (20:5, n-3), inhibit tumorigenesis (5, 6), growth of rodent tumors (7, 8), and human breast cancer xenografts (9, 10). Importantly, it has been reported that n-3 PUFAs selectively inhibit tumor cell proliferation and are significantly less toxic toward normal cells (11, 12). Human tumor cells differ in their sensitivity to n-3 PUFAs, which cannot be explained simply by differences in fatty acid uptake (13) but may be related to the level of expression of antioxidant enzymes (14).

The mechanisms responsible for the antitumor effects of n-3 PUFAs have not been completely defined, but published evidence supports the hypothesis that lipid peroxidation is a crucial part of their action (15). The susceptibility of n-3 PUFAs to lipid peroxidation renders them capable of rapidly generating lipid peroxides, which may directly cause cytotoxicity or may influence intracellular signaling pathways, resulting in growth inhibition or death of tumor cells (15). This hypothesis is supported by experiments showing that n-3 PUFA effects can be attenuated or reversed completely by the antioxidant, vitamin E (15, 16). Moreover, the cytotoxicity of n-3 PUFAs on malignant cells can be accelerated by increasing cellular oxidative stress (17).

The balance between oxidants and antioxidants is critical for a biological system to maintain its normal function. Antioxidant defenses are provided by both small molecules such as glutathione, vitamin E, and vitamin C and enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx; ref. 18). There are several forms of SOD in eukaryotic cells, which differ in their metal binding characteristics and intracellular distribution. Cu/Zn-SOD, also called SOD1, is located mainly in the cytoplasm, comprises 90% of the total cellular SOD (19), and requires Cu and Zn for its activity (20). A portion of SOD1 protein is also found in the mitochondria in eukaryotic cells (19, 21–23). Mn-SOD, also called SOD2, is located primarily in the mitochondrial matrix and requires Mn for its activity. A third type of SOD, found in the extracellular space, has also been reported (24). At least five forms of GPx have been cloned (25–29), whereas no isoforms of catalase have been reported in humans. It has been shown that antioxidant enzyme levels are altered in cancer (30, 31). The most consistently reported finding is that expression of SOD2 is lower in most types of cancers (30) and it has been suggested that SOD2 is a tumor suppressor gene. Because...
Cytotoxicity of DHA and the Role of SOD1

all cells actively produce superoxide during routine metabolic processes, the reduced expression of SOD2 by cancer cells (32–34) suggests that malignant cells may be more dependent for survival on other antioxidant enzymes than normal cells.

The present study has examined the cytotoxic effects of DHA on five different human cancer cell lines and explored the potential cellular mechanisms explaining the different sensitivity among those cell lines. We show that a transformed large cell lymphoma cell line (DHL-4) is highly sensitive to DHA treatment and that, in these cells (but not in DHA-resistant lines), DHA alters the expression of SOD1, which suggests a mechanism that may contribute to the differential sensitivity of tumor cells to DHA.

Materials and Methods

CellTiter 96 AQueous One Solution Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay] reagent was from Promega (Madison, WI). DHA and eicosapentaenoic acid were from Sigma Chemical Co. (St. Louis, MO). SuperScript II was from Invitrogen (Carlsbad, CA). Taq polymerase was from Roche (Indianapolis, IN). Antibodies for SOD1 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were analytic grade.

Cell Culture and Cell Viability Assay

The human B-cell lymphoma line, DHL-4, was a generous gift from Dr. Linda Boxer (Stanford University, Stanford, CA). The cervical cancer line, SiHa, was kindly provided by Dr. Doris Benbrook (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The human breast cancer lines (MCF-7 and MDA-MB-231), a human breast endothelia line (MCF-10A), and a B-cell lymphoblastoid line (Raji) were obtained from American Type Culture Collection (Manassas, VA). MCF-7, MDA-MB-231, and Raji, and DHL-4 cells were cultivated in RPMI 1640 supplemented with 2 mmol/L l-glutamine and 10% FCS. SiHa cells were cultivated in DMEM supplemented with 2 mmol/L l-glutamine and 10% FCS. MCF-10A cells were maintained in 1:1 mixture of DMEM and Ham’s F12 medium supplemented with 10% horse serum, l-glutamine, human recombinant epidermal growth factor, insulin, cholera toxin, and hydrocortisone. Cells were routinely grown in 75 cm² flasks in an environment containing 5% CO₂ and passaged every 3 days. Cell growth was analyzed using the MTS assay. Cells (1,000–4,000, but 10,000–20,000 for the lymphoid lines) were plated in each well of a 96-well tissue culture plate in 100 μL medium. This guaranteed a 40% to 50% confluence of the cells after 24 hours of growth. The medium was replaced with 100 μL fresh medium containing DHA or other reagents and the cells were grown for designed periods of time. Stock DHA was dissolved in ethanol and mixed with bovine serum albumin at 4:1 molar ratios before adding to cells. Control cells were treated with ethanol mixed with bovine serum albumin as vehicle. MTS assay procedures recommended by the manufacturer were used. In brief, 20 μL MTS solution was added to each well and cells were incubated at 37°C for 1 to 2 hours and the absorbance was read at 490 nm. Data are presented as percentages of values detected in control cells cultured under the same conditions.

Apoptosis/Necrosis Assay

Hoechst/propidium iodide nuclear staining and fluorescence microscopy, a well-established technique for the morphologic assessment of cell death (35, 36), was used. Cells were cultured with Hoechst dye (5 μg/mL, final concentration) for 15 minutes at 37°C, centrifuged at 1,000 rpm for 5 minutes, and washed once with PBS. Pelleted cells were resuspended in PBS at 2 × 10⁶ cells/mL. Propidium iodide (50 μg/mL, final concentration) was added and the cells analyzed with fluorescence microscopy using a scoring system described previously (35, 36). In brief, “viable” cells had blue-stained, smooth nuclei; “viable apoptotic” cells had blue-stained nuclei with multiple bright specks of condensed chromatin; “nonviable apoptotic” cells contained red-stained nuclei with either multiple bright specks of fragmented chromatin or one or more spheres of condensed chromatin; and “necrotic” cells had red-stained, smooth nuclei that were nearly the same size as normal nuclei. At least 200 cells were counted for each experiment.

DNA Fragmentation Assay

Cells were plated in a six-well plate (200,000/well) and treated with 30 mmol/L DHA for 16 hours. The cells were pelleted by centrifugation and resuspended by vigorous vortexing in 500 μL buffer containing 0.5 mol/L EDTA, 10 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 9), and 1% SDS and incubated at 50°C for 2 hours. Samples were extracted with equal volume of water-saturated phenol/chloroform (3:4, v/v) twice, and phenol was removed from the aqueous phase by one extraction with chloroform. DNA was precipitated by addition of 2.5 volumes of ethanol and NaCl to 0.1 mol/L to final concentration overnight at −20°C. After centrifugation at 12,000 rpm at 4°C for 30 minutes, the pelleted DNA was washed once with 70% ethanol and resuspended in 10 mmol/L Tris-EDTA buffer. DNA (~10 μg) was loaded in each well of a 1.5% agarose gel and subjected to electrophoresis using a Tris-acetate-EDTA buffer. DNA was stained with ethidium bromide and visualized under UV light.

Thiobarbituric Acid Reactive Substance Assay

The assay protocol developed by Erdahl et al. (37) was used with minor modifications. Cells were grown in 75 cm² flasks until 80% confluent and incubated with DHA for 6 or 20 hours. After harvesting, cells were suspended in 200 μL PBS and sonicated for 15 seconds. The sonicated material (150 μL) was added to 1.5 μL solution containing 100 mmol/L butylated hydroxytoluene. Fifteen percent trichloroacetic acid (75 μL), 0.25 mol/L HCl (75 μL), 0.25 mmol/L butylated hydroxytoluene (75 μL), 0.375% thiobarbituric acid (75 μL), and 8.5% SDS (30 μL) were added to the sample and vortexed for 1 minute. The color was
developed at 95°C for 60 minutes and the reaction was stopped by cooling on ice for 10 minutes. The samples were centrifuged at 1,500 rpm for 10 minutes and the supernatant (200 μL) from each tube was transferred to a 96-well plate. The absorbance at 540 nm was measured with reference to a reagent blank. Thiobarbituric acid reactive substance (TBAR) was calculated from a standard curve made with various concentrations of 1,1,3,3-tetraethoxypropane and normalized to the protein concentration (determined by protein assay reagent, Bio-Rad, Hercules, CA) of each sample.

**Reverse Transcription-PCR**

Cells were grown and treated in six-well plates (DHL-4, 200,000 cells/well; SiHa, 40,000 cells/well). Total RNA was isolated by adding TRIzol reagent (500 μL, Invitrogen) to each well. After incubation for 5 minutes at room temperature, chloroform (100 μL) was added and the mixture was centrifuged. The aqueous phase was collected and the DNA was precipitated with isopropanol. It was washed once with 70% ethanol and air-dried. Reverse transcription was carried out at 42°C for 40 minutes in 20 μL mixture containing 5 μg RNA, 200 units SuperScript II, 40 units RNase inhibitor, 0.5 mmol/L of each deoxynucleotide triphosphate, and 100 ng oligodeoxythymidylic acid (dT) for 1 hour. The RNA was precipitated with isopropanol. It was washed once with 70% ethanol and air-dried. Reverse transcription was carried out at 42°C for 40 minutes in 20 μL mixture containing 5 μg RNA, 200 units SuperScript II, 40 units RNase inhibitor, 0.5 mmol/L of each deoxynucleotide triphosphate, and 100 ng oligodeoxythymidylic acid primers followed by incubation at 70°C for 15 minutes. The primers used for PCR are shown in Table 1. We have evidence indicating that the amplification of the templates cDNA by using these primers is in a log-phase increase between 25 and 40 cycles of PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. The PCR reaction contained 1 μL cDNA, 0.2 μmol/L sense and antisense primers, 0.4 mmol/L deoxynucleotide triphosphate mix, 1.5 mmol/L Mg²⁺, and 2.5 units Taq polymerase in a 25 μL volume. The thermal cycles used included cDNA denaturation at 95°C for 3 minutes followed by 30 cycles at 94°C for 1 minute, 51°C for 2 minutes, and 72°C for 3 minutes. Elongation was done at 72°C for 12 minutes. The PCR reaction products were separated in a 1% agarose gel containing ethidium bromide and visualized under UV light. The expression level of genes was analyzed with densitometry and normalized to that of GAPDH.

**Small Interfering RNA Procedures**

Sequence information regarding mature human SOD1 mRNA was extracted from the National Center for Biotechnology Information Entrez nucleotide database (Bethesda, MD). Two target sites within the gene were chosen from the mRNA sequence of SOD1 (Genbank accession no. BC001034). Following selection, each target site was searched with National Center for Biotechnology Information BlastN to confirm their specificity. Two different small interfering RNAs (siRNA) were designated SOD1-siRNA(a) and SOD1-siRNA(b), which target nucleotides 148 to 166 and 279 to 298 of the human SOD1 mRNA sequence, respectively. The 64-mer sense and antisense oligonucleotide templates (19 × 2 nucleotides specific to the targeted genes and 26 nucleotides for restriction enzyme sites and hairpin structure) were synthesized by Integrated DNA Technologies (Corvalle, IA; ref. 38). The DNA oligonucleotides were annealed, phosphorylated, and ligated to pUB/Bsd/H1 vector at BamHI/HindIII sites. This vector was provided by Dr. Leonidas Tsiokas (University of Oklahoma Health Sciences Center) and contains the blasticidin resistance gene. Successful ligation was confirmed by enzyme digestion and by direct sequencing. The constructs [4 g of the mixture of SOD1-siRNA(a) and SOD1-siRNA(b)] were transfected into SiHa cells using LipofectAMINE Reagent (Invitrogen). Three days after transfection, the cells were selected by the addition of blasticidin (10 μg/mL, final concentration) to the medium for 6 days. The selected cells were immediately subjected to reverse transcription-PCR or viability assay experiments.

**SOD1 cDNA Cloning and Overexpression in DHL-4 Cells**

SOD1 cDNA was PCR amplified from MDA-MB-231 cells using the following primers: sense 5'-CCGGTATTAGGCGATCCCAAT-3' and antisense, 5'-CCGGTATTAGGCGATCCCAAT-3'. The amplified fragment was ligated into pcDNA3 vector at KpnI sites. DNA transfections were done using electroporation as described previously (39). In brief, DHL-4 cells in log phase (5 × 10⁵–6 × 10⁵ cells/mL) were washed with RPMI 1640 and resuspended at 3 × 10⁵ cells/mL in RPMI 1640 containing 25 g/mL DEAE-dextran and 15 g plasmid DNA, and electroporation was done with a Bio-Rad Gene Pulser at 960 F and 320 mV. Transfected cells were cultured overnight in RPMI 1640 with supplements. The cells were plated in 96-well plate at 20,000/well.

**Western Blot Analysis**

DHL-4 cells were lysated in buffer containing 50 mmol/L Tris-HCl, 100 mmol/L NaCl, 5 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton, and 1% sodium deoxycholate and sonicated on ice. The lysates were clarified by centrifugation at 10,000 × g for 15 min. The resulting supernatant was used for protein analysis by Western blotting. The blots were probed with rabbit polyclonal antibodies to SOD1 or GAPDH and visualized with an enhanced chemiluminescent detection system (Amersham). The expression level of SOD1 was measured by densitometry and normalized to that of GAPDH.

**Table 1. Primers used for PCR amplification**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Accession No. / Amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1-F</td>
<td>5'-CTAGCGAGTTATGGCGAC-3'</td>
<td>BC001034, 58/538, 481</td>
</tr>
<tr>
<td>SOD1-R</td>
<td>5'-AGATGTATTGCGAGATC-3'</td>
<td></td>
</tr>
<tr>
<td>GPx-1-F</td>
<td>5'-CACCCCGAGATGACAAGCAG-3'</td>
<td></td>
</tr>
<tr>
<td>GPx-1-R</td>
<td>5'-CGCCAGAGATGCGACAG-3'</td>
<td></td>
</tr>
<tr>
<td>Catalase-F</td>
<td>5'-CAGCCAGAGATGCGACAG-3'</td>
<td></td>
</tr>
<tr>
<td>Catalase-R</td>
<td>5'-ATCCAGGAGGCTTCAAGGAC-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>5'-TGCGGAAAGTGAAGGTCGG-3'</td>
<td>BC029618, 56/300, 245</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>5'-GGAGATCTCGCTCCTGGAAG-3'</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Sense and antisense primers used for the amplification of SOD1, GPx-1, catalase, and GAPDH cDNA.
2.5% glycerol. The lysated cells were sonicated and incubated at 4°C for 1 hour and centrifuged at 13,000 rpm for 30 minutes. The supernatant was collected and protein concentration was determined. Protein (20 μg) from each sample was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was blocked with 5% milk in TBST and immunobloted with primary antibody at 4°C for overnight and secondary antibody at room temperature for 1 hour. Chemiluminescence was used for signal detection.

**Results**

**Effects of DHA on Cell Viability of Human Cancer Cell Lines**

We first examined the effects of DHA on the viability of five different human tumor cell lines. Treatment with 30 μmol/L DHA for 72 hours had little effect on the growth of two breast cancer cell lines (MCF-7 and MDA-MB-231) or a cervical cancer cell line (SiHa). DHL-4 cells (a transformed, large B-cell lymphoma line carrying the 14:18 translocation, which overexpresses bcl-2; ref. 40) were very sensitive to DHA treatment for the same period of time (Fig. 1). A moderate (30%) reduction of cell viability was seen in the Burkitt’s lymphoma line, Raji, which is similar to previous findings (41). At high concentration of DHA (300 μmol/L), viability of MCF-7, MDA-MB-231, and SiHa cell lines were inhibited to a similar extent, with SiHa cells being the most resistant. Detailed analysis revealed that the IC₅₀ of DHA for DHL-4 cells is 5.2 mol/L and that for SiHa cells is >300 μmol/L. Time course studies indicated that the cytotoxic effect of DHA on DHL-4 cells could be detected as early as 24 hours after addition of the fatty acid and that 48 hours were required for DHA to kill most of the cells (Fig. 2). DNA ladder assay and morphologic assessment on DHL-4 cells were done to determine whether apoptosis is involved in DHA-induced cell death. Figure 3 shows that treatment with 30 μmol/L DHA for 16 hours causes cell death of DHL-4 cells primarily through an apoptotic pathway (Fig. 3). To our knowledge, such sensitivity to DHA has not been reported in any cancer cell lines studied previously. Doxorubicin, a chemotherapeutic agent used clinically, had similar cytotoxic effects on both DHL-4 and SiHa cells (data not shown). Because DHL-4 cells were the most sensitive and SiHa cells were the most resistant to the cytotoxic effect of DHA, we focused our attention on these two cell lines.

**Involvement of Lipid Peroxidation in DHA-Mediated Cytotoxicity**

It has been shown in previous study that the cytotoxic effect of n-3 PUFAs on tumor cells requires lipid peroxidation (15). We therefore used the TBAR assay to measure lipid peroxidation following DHA treatment of DHL-4 and SiHa cells (Fig. 4). Treatment with DHA caused a 3.5-fold increase in TBAR in DHL-4 cells but not in SiHa cells. To further confirm the involvement of membrane lipid peroxidation in this event, we pretreated DHL-4 cells with increasing concentrations of α-tocopherol (a lipophilic compound known to block membrane peroxidation) and Trolox (a short-chain, water-soluble analogue of α-tocopherol, known to have more potent cytoprotective effect than -tocopherol; ref. 42) before addition of 30 μmol/L DHA. As shown in Fig. 5, 10 μmol/L -tocopherol was sufficient to completely reverse cytotoxicity caused by 30 μmol/L DHA,

![Figure 1](https://example.com/image1.png)  
*Figure 1.* Sensitivity of tumor cell lines to DHA-induced cytotoxicity. Tumor cell lines were plated in 96-well plates and exposed to various concentrations of DHA for 72 hours. Cell viability was analyzed using the MTS assay. No surviving DHL-4 cells were present at higher DHA concentrations. Columns, means from three independent experiments; bars, SEM.
whereas 30 μmol/L Trolox was required to have the same effect. These studies confirmed that membrane lipid peroxidation was responsible for the cytotoxic effect of DHA.

**Expression of SOD1, GPx-1, and Catalase in SiHa and DHL-4 Cells**

The expression of the primary cellular antioxidant enzymes SOD1, GPx-1, and catalase was examined to understand if they are related to the differential sensitivity of tumor cells to DHA. The mRNA expression of SOD1, GPx-1, and catalase was detected in both cell lines as measured with reverse transcription-PCR (Fig. 6A). The expression of GAPDH was also measured as an internal control to insure that similar amounts of RNA were loaded in each lane. Treatment with 30 μmol/L DHA for 16 hours had no effect on the expression of these enzymes in SiHa cells but resulted in a 53% decrease in SOD1 mRNA level in DHL-4 cells and this was confirmed at protein level by Western blot technique (Fig. 6B). To confirm the involvement of SOD1 in DHA-induced cytotoxicity, a SOD1 cDNA construct was established. Overexpression of SOD1 in DHL-4 cells (Fig. 7A) resulted in attenuated DHA effects on cell viability (Fig. 7B).

**Effects of Knockdown of SOD1 on DHA-Induced Lipid Peroxidation and Cytotoxicity in SiHa Cells**

Because SiHa was the line most resistant to DHA treatment and did not down-regulate SOD1 expression in response to DHA, we hypothesized that SOD1 might be particularly important in explaining its resistance to DHA. We therefore used siRNA to decrease SOD1 expression of SiHa cells. As shown in Fig. 8A, expression of SOD1 was significantly down-regulated by siRNA (75%) as measured by the sensitive semiquantitative PCR assay. Down-regulation of SOD1 did not affect the growth of SiHa cells (Fig. 8B) but did result in a significant increase (3.2-fold, Fig. 9A) in TBAR generation following treatment.
with DHA and in greater cytotoxicity (Fig. 9B). SOD1 down-regulation augmented DHA cytotoxicity in these cells, suggesting that the level of SOD1 expression contributes to the sensitivity of tumor cells to DHA-induced cytotoxicity.

Effects of DHA on Human Breast Endothelia Cells (MCF-10A) and Breast Cancer Cells (MCF-7)

Because DHA has been shown to have different cytotoxic effects on MCF-7 and MCF-10A cells (43), we choose these two cell lines to test whether the different sensitivity of DHA on normal and tumor cells is also related to SOD1 expression. As shown in Fig. 10, DHA-induced cytotoxicity was significantly higher in MCF-7 cells than in MCF-10A cells, similar to previous report (43). However, DHA did not alter SOD1 expression in both cell lines (data not shown), suggesting that the selective effect of DHA on tumor cells over normal cells may not be related to SOD1 expression.

Discussion

n-3 PUFA–induced cytotoxicity toward human lymphoma cell lines has been reported previously (44, 45). Anel et al. first reported that DHA is toxic for Raji cells but not for normal human lymphocytes (44, 45). Subsequent studies confirmed that DHA causes both apoptosis and necrosis in Raji and the related Ramos cells (41) and that eicosapentaenoic acid–induced apoptosis depends on acyl-CoA synthetase (44, 45). The effects of n-3 PUFAs on DHL-4 cells have not been studied previously. An interesting finding from the present study was that DHL-4 cells are highly sensitive to the cytotoxic effect of DHA (IC50, 5.2 mol/L), with effects evident within 24 hours. The cell death induced by DHA was primarily through the apoptotic pathway. Although the cytotoxic effects of DHA toward tumor cells have been extensively studied, to our knowledge, the DHL-4 cell line is the most sensitive line studied to date. As such, it may provide an optimal model system for investigating mechanisms of cytotoxicity and resistance to n-3 PUFAs.

It has been shown previously that tumor cells display different sensitivities to n-3 PUFAs (41), but the exact mechanisms responsible were not delineated. Because others have shown that lipid peroxidation may be the key mechanism underlying n-3 PUFA–induced cytotoxicity (15),

Figure 6. Effects of DHA on the expression of SOD1, GPx-1, and catalase in DHL-4 and SiHa cells. Total RNA was isolated from SiHa and DHL-4 cells, reverse transcribed, and PCR amplified using specific primers. The products of the PCR reactions were separated on 1% agarose gel and stained with ethidium bromide. Data are representative of three experiments. SOD1 protein expression was determined by Western blotting technique using specific antibody. A, expression of SOD1, GPx-1, and catalase in the presence and absence of 30 μmol/L DHA for 16 hours in SiHa and DHL-4 cells. B, mRNA and protein expression of SOD1 in the presence and absence of 30 μmol/L DHA for 1 and 16 hours in DHL-4 cells. Top, densitometry of DHA-induced decrease in SOD1 mRNA level. * P < 0.05, relative to control cells (one-way ANOVA).

Figure 7. Overexpression of SOD1 in DHL-4 cells attenuates DHA-induced cytotoxicity. DHL-4 cells were transfected with SOD1 cDNA constructs using electroporation technique. SOD1 was measured at protein level 72 hours after transfection (A). The cells were treated with DHA for 48 hours and cell viability was analyzed using the MTS assay (B). Data are representative of two separate transfection experiments.
we studied both a sensitive (DHL-4) and a resistant (SiHa) line in an attempt to determine whether differences in elements related to lipid peroxidation explain the differing sensitivities of cells to DHA.

Several lines of evidence presented in this study indicate that differences in sensitivity to DHA exposure is determined to a large extent by the level of lipid peroxidation that is caused by DHA. First, treatment with DHA caused a 3.5-fold increase in the level of lipid peroxidation (measured with the TBAR assay) in the sensitive DHL-4 cells and no increase in the resistant SiHa cells. Second, pretreatment with vitamin E, an antioxidant known to attenuate lipid peroxidation, completely blocked DHA-mediated cytotoxicity. In addition, the protection afforded by α-tocopherol (a lipophilic antioxidant) was greater than that obtained from pretreatment with Trolox (a water-soluble compound more likely to partition to the cytoplasm). Third, treatment with DHA caused a decrease in the expression of SOD1 in the sensitive DHL-4 cells but not in the resistant SiHa cells. This suggests that SOD1 expression may be an important determinant of the vulnerability of cells to DHA. Indeed, overexpression of SOD1 in DHL-4 cells attenuated DHA cytotoxicity. Furthermore, targeting SOD1 expression by siRNA in SiHa cells resulted in both increased lipid peroxidation (as assessed by TBAR generation) and increased sensitivity to the cytotoxic effects of DHA.

Others have examined the effects of fatty acids on the activity of antioxidant enzymes in vivo, with conflicting results. Some studies have reported an increase in the activity of these enzymes in animals fed PUFA-enriched diets (46–49). Others have shown that n-3 PUFAs reduce the activity of antioxidant enzymes in noncancerous rat tissues (50–52). In the present study, we found that DHA selectively down-regulates SOD1 expression in DHL-4 cells, which has not been reported previously. DHA is known to modulate expression of genes in tumor cells (53–55). Transport of fatty acids such as DHA to the nucleus and binding to nuclear receptors such as peroxisome proliferator-activated receptor have been reported (56, 57). Of note is the report of a peroxisome proliferator-activated receptor response element in the promoter of the rat SOD1 gene (58). Transcription of the SOD1 gene is regulated differentially.

Figure 8. siRNA-mediated knockdown of SOD1 expression in SiHa cells. SOD1 expression in SiHa cells was knocked down using siRNA, as described in Materials and Methods. A, reverse transcription-PCR and densitometry analysis of SOD1 (78% down-regulation) expression. B, the growth rate of SiHa cells is not affected by knockdown of SOD1 expression. Columns, mean (n = 3) absorbance (490 nm) of the cells following the addition of the MTS reagent; bars, SEM.

Figure 9. Effects of knockdown of SOD1 on DHA-induced lipid peroxidation and cytotoxicity in SiHa cells. A, SiHa cells exhibiting decreased SOD1 expression (or control cells transfected with an empty vector) were treated with 30 μmol/L DHA for 20 hours following which generation of TBAR was determined. Data are representative of two separate experiments. B, SiHa cells exhibiting decreased SOD1 expression (or control cells transfected with an empty vector) were treated with 30 and 100 μmol/L DHA for 72 hours. Cell viability was analyzed using the MTS assay. Columns, means from three independent experiments; bars, SEM. *, P < 0.05, relative to control cells (one-way ANOVA).
by DHA in DHL-4 and SiHa cells and may account for the selective effects of DHA on SOD1 mRNA levels. Alternatively, DHA may destabilize the SOD1 mRNA, leading to lower expression. Targeting SOD1 has been suggested previously as a means of increasing intracellular superoxide levels, thereby increasing mitochondrial injury and inducing apoptosis in cancer cells (59). Interestingly, complete knockdown of SOD1 expression by siRNA induces senescence in human fibroblast and causes cell death in malignant HeLa cells (60). In our experiments, we achieved 75% knockdown of SOD1 expression in SiHa cells. This did not alter the growth rate of cells but had a significant effect on DHA-induced lipid peroxidation and cytotoxicity. These results support the idea that drugs that alter SOD1 expression or function may prove to be useful in sensitizing tumor cells to some chemotherapeutic drugs.

This report provides the basis for additional studies into the mechanisms underlying n-3 PUFA–induced cytotoxicity. First, it identifies a particularly sensitive cell line. Second, it shows an acute effect of DHA administration on expression of an important cellular antioxidant enzyme. Third, it shows that down-regulation of SOD1 can alter the sensitivity of a cell to a member of an important group of essential fatty acids. Finally, it ties together membrane lipid peroxidation and gene expression as important, interrelated mechanisms underlying cellular sensitivity to n-3 PUFAs.

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Figure 10. Effects of DHA on the viability of MCF-7 and MCF-10A cells. MCF-7 and MCF-10A cells were treated with increasing concentrations of DHA for 72 hours. Cell viability was analyzed using the MTS assay. Columns, means from two independent experiments; bars, SEM.


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Molecular Cancer Therapeutics

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