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Modulation of Inducible Nitric Oxide Synthase and Related Proinflammatory Genes by the Omega-3 Fatty Acid Docosahexaenoic Acid in Human Colon Cancer Cells[1]

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ABSTRACT

Epidemiological and preclinical studies demonstrate that consumption of diets high in omega-3 polyunsaturated fatty acids reduces the risk of colon cancer. Inhibition of colon carcinogenesis by omega-3 polyunsaturated fatty acids is mediated through modulation of more than one signaling pathway that alters the expression of genes involved in colon cancer growth. In our earlier studies on global gene expression with cDNA microarrays, we have shown that treatment of CaCo-2 colon cancer cells with docosahexaenoic acid (DHA) down-regulated the prostaglandin family of genes, as well as cyclooxygenase 2 expression and several cell cycle-related genes, whereas it up-regulated caspases 5, 8, 9, and 10 that are associated with apoptosis. It is known that nitric oxide activates the cyclooxygenase 2 enzyme, which plays a pivotal role in the progression of colon cancer via prostaglandin synthesis and angiogenesis. The present study was undertaken to examine the multifaceted role of DHA in the expression of inducible nitric oxide synthase (iNOS) and of related proinflammatory genes, as those have been shown to play a role in tumor progression. In addition, we aimed to identify associated target genes by DNA microarray, reverse transcription-PCR analysis, and cellular localization of iNOS expression in CaCo-2 cells. Results of this study demonstrate that treatment with DHA down-regulates iNOS in parallel with a differential expression and down-regulation of IFNs, cyclic GMP, and nuclear factor κB isoforms. More importantly, our findings clearly demonstrate the up-regulation of cyclin-dependent kinase inhibitors $p21^{(Waf1/Cip1)}$ and p27, differentiation-associated genes such as alkaline phosphatases, and neuronal differentiation factors. These finding strongly suggest that the antitumor activity of DHA may be attributed, at least in part, to an effect on iNOS regulatory genes. In addition, our results indicate the presence of specific gene expression profiles in human colon cancer that can be used as molecular targets for chemopreventive agents.

INTRODUCTION

Cancer of the colon is one of the leading causes of deaths in both men and women in Western countries, including the United States, where ~148,300 new cases of colorectal cancer and 56,600 related deaths are expected for the year 2002 (1). Epidemiological studies have provided evidence that high intake of saturated fat and/or animal fat increases the risk of colon cancer, and that diets rich in omega-3 PUFAs[3] (fish oils) reduce the risk of colon cancer development (2, 3). Caygill *et al.* (2) reported an inverse association of consumption of fish and fish oil with colon cancer. Studies in our laboratory and elsewhere have provided convincing evidence that diets rich in omega-3 PUFAs reduce the risk of chemically induced colon carcinogenesis compared with diets high in omega-6 PUFAs and/or saturated fatty acids. This suggests that the composition of the ingested fat is critical to colon cancer risk (4–7). In addition, laboratory animal assays have indicated that the influence of these omega-3 PUFAs is exerted foremost during the postinitiation phase of colon carcinogenesis (5). In a Phase II clinical trial of patients with colonic polyps, dietary fish oil supplements have, in fact, inhibited cell proliferation in the colonic mucosa (8).

With regard to mode of action of different types of fat in colon carcinogenesis, dietary fish oil decreases the concentration of secondary bile acids in the colon as compared with diets high in omega-6 PUFAs and saturated fats (9). Secondary bile acids have been shown to increase cell proliferation and to act as colon tumor promoters (10, 11). However, the cellular and molecular mechanisms by which omega-3 PUFAs inhibit colon carcinogenesis and reduce the growth of tumor cells remain poorly understood. Preclinical evidence demonstrated that several dietary components could influence the pathways involved in cell proliferation and differentiation (12, 13). Our earlier studies demonstrated that omega-3 fatty acids are capable modulating a panel of cell cycle and apoptosis-regulating genes in tumors (14). Much attention has been given recently to endogenous factors that appear to be responsible for tumor cell growth, metastasis, and invasion. Identifying whether such endogenous factors are modulated by omega-3 PUFAs should lead to a better understanding of the processes of tumor cell progression, and would also provide new strategies for developing nutritional and chemopreventive agents that specifically suppress these processes.

Preclinical model assays indicate that dietary fish oil inhibits COX-2 activity and enhances apoptosis in colon tumors (7). Overexpression of the *COX-2* gene in colonic epithelial cells leads to altered adhesion properties and resistance to apoptosis (14). Although the above studies are indirectly supporting the anticancer properties of the omega-3 fatty acid DHA (Fig. 1), a thorough understanding of the pathways that are involved in the mechanism of tumorigenesis is necessary to fully assess the chemopreventive efficacy of this agent against colon cancer. In this connection we have demonstrated recently by cDNA microarray and RT-PCR analyses that in CaCo-2 cells DHA alters several proinflammatory genes (15). Studies in our laboratory and elsewhere support the hypothesis that COX-2 regulation is influenced by various exogenous factors including NO (16–19). Remarkably, several studies have demonstrated that colonic tumors in laboratory animals, and colonic adenomas and adenocarcinomas in humans have increased activities and/or expression of iNOS when compared with the levels in adjacent non-neoplastic mucosa (19–22). High levels of iNOS may increase the invasiveness and metastatic potential of human colon cancer (23). It is also known that NO can damage DNA either directly or indirectly by several mechanisms including interference with DNA repair. NO can also cause post-translational modifications of proinflammatory cytokines that may lead to tumor initiation and promotion (24, 25). Therefore, it is possible that sustained high levels of NO generated by iNOS can produce various kinds of damage. In chronic conditions, such damage leads to an accumulation of gene mutations, including mutation of the tumor suppressor gene *p53*, and alterations in cellular functions (26). Taken together, these observations suggest that *COX-2*, iNOS, and

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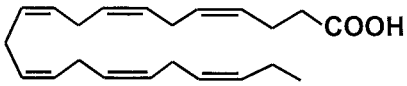
[3] The abbreviations used are: PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription-PCR; RXR, retinoic acid X receptor; STAT, signal transducers and activation of transcription; NO, nitric oxide; DAPI, 4′,6-diamidino-2-phenylindole; NFκB, nuclear factor κB.



Docosahexaenoic acid

Fig. 1. Chemical structure of DHA.

other proinflammatory genes may play a critical role in colon carcinogenesis.

In view of the significance of omega-3 PUFAs, including DHA, in colon carcinogenesis, and involvement of COX-2 and iNOS in colon tumor progression, the current study was designed to explore the mechanisms of chemopreventive efficacy of DHA, specifically on modulation of iNOS and other proinflammatory genes with respect to apoptosis and cell differentiation. First, we determined the effect of DHA on colon cancer cell growth, apoptosis, and DNA fragmentation, and then its effect on iNOS expression by nuclear localization with iNOS-specific antibodies and by Western blot for the iNOS proteins. Also, we identified COX-2- and iNOS-activating proinflammatory genes, and their levels of expression in DHA-treated CaCo-2 cells using cDNA microarray analysis. Although NO functions as a mediator in the inflammatory processes while it also is a physiologically important signaling molecule in virtually every tissue in the body, we addressed here the question of whether DHA could indeed induce cell death via down-regulation of iNOS expression and/or by modulating sets of genes involved in apoptosis and differentiation.

MATERIALS AND METHODS

Cell Growth and DHA Treatment. CaCo-2 cells were grown under cell culture conditions and maintained by serial passage in RMPI 1640 containing 10% FBS. For stimulation, DHA (Cayman, Ann Arbor, MI) was dissolved in DMSO, and cells were treated with 5 μg/ml of DHA in the cell culture medium for 48 h. Control cultures were treated with DMSO alone and were processed similarly.

RNA Isolation and Probe Preparation for Microarray Analysis. Untreated CaCo-2 cells and those treated with DHA for 48 h were collected, and total RNA was isolated using TriZol reagent and Qiagen columns (Life Technologies, Inc. Rockville, MD and Qiagen, Valencia, CA, respectively). One control probe (untreated CaCo-2 cells) and one test probe (DHA-treated CaCo-2 cells) were made independently for microarray hybridization. RNA from the untreated cells was labeled with Cy3 and used as the control probe. RNA from DHA-treated CaCo-2 cells was labeled separately with Cy5 and was used as the test probe. The reverse transcription reaction was carried out, and the labeled probes were washed with 70 and 95% ethanol, respectively, and were stored at −20°C for additional hybridization. Hybridizations were carried out as described earlier (15).

Human Oligonucleotide Array. The impact of DHA on gene expression profiles was performed using Clontech Human Atlas Glass Arrays. Each gene on Atlas Glass Arrays is represented by a "long oligo," an 80-bp fragment, which has 70% homology to any entry in GenBank that combines the high hybridization efficiency of a cDNA fragment with the ability of a short oligonucleotide to distinguish between homologous genes. Atlas Glass 3.8 microarrays contain 3800 carefully selected, well-characterized genes to provide high-quality, reliable expression data from many biological pathways. Briefly, the genes on the array include a number of functional categories of genes and transcription factors relevant to this study.

Scanning and Image Analysis. Microarray slides were scanned using an Axon GenePix 4000A scanner (Axon Instruments, Foster City, CA). This is a nonconfocal scanning instrument containing two lasers that excite cyanine dyes at appropriate wavelengths, 635 nm for Cy5 and 532 nm for Cy3, respectively, with high-resolution (10 μm pixel size) photo multiplier tubes that detect fluorochrome emission. The photo multiplier tube levels of the two channels at 635 nm and 532 nm were balanced (100–1000 V) to limit the number of saturated pixels for generating a gray scale TIFF image file. The microarray images were analyzed using GenPix Pro-3.0 software. The microarray data sets and color images were generated on Microsoft Excel spreadsheets and JPEG images, respectively. The GeneSpring bioinformatics software package (Silicon Genetics, Inc.) was used to explore the microarray data sets generated from this study for multivariate analysis.

Validation of Gene Expression by RT-PCR. Because RT-PCR of mRNA provides maximum sensitivity, a standardized measurement of expressed genes was carried out by a semiquantitative RT-PCR. The RT-PCR used 33 cycles for selected gene-specific primer sequences. All of the templates were initially denatured for 2 min at 94°C, and the amplification of the amplicon was extended at a final extension temperature of 72°C for 7 min. A separate set of RT-PCR reactions with an increasing amount of RNA was carried out, if necessary, to show a linear increase in the band intensity of the amplified PCR product. PCR amplification with glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Cellular Localization of iNOS. In this study, we have used nuclear staining of colon cancer cells for detecting iNOS-positive cells by immunofluorescence technique based on published results of Fehr *et al.* (27) indicating that the receptors of certain cytokines signal through STAT proteins. Receptor occupation and dimerization induce phosphorylation of STATs. Activated STATs dimerize and translocate to the nucleus where they increase the expression of transcription factor 1RF-1, which binds to specific DNA elements in the iNOS gene promoter region to increase iNOS gene expression. We have detected this gene by nuclear staining as described here. CaCo-2 cells with or without 48 h of DHA treatment were fixed in 10% formalin and pretreated with 0.1% Triton X-100 and 2 N HCl at 37°C for 10 min. They were then treated with 0.1 M sodium borate for 5 min and washed with PBS three times. Immunofluorescence detection of iNOS-positive cells was visualized with anti-iNOS antibody (Cayman) followed by rhodamine conjugated with goat-antimouse IgG. An epifluorescence microscope (AX-70; Olympus, Tokyo, Japan) was used for detection of iNOS-positive cells. The positively stained cells were quantified with Image Pro plus software (Media Cybernetics, Silver Spring, MD).

Western Blot Analysis for iNOS Expression. CaCo-2 cells treated with DHA (10^{-5} M) for 48 h were harvested by trypsinization. Cellular protein was isolated with protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS in addition to a mixture of protease inhibitors (Boehringer Mannheim, GmbH, Germany). Equal amounts of protein (50 μg/lane) were fractioned on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The Western blot procedure was carried out as described earlier (26). The antibody used for Western blotting was iNOS polyclonal antiserum (Cayman). The reactive protein band for iNOS expression was developed using chemiluminescent detection reagents (ECL; Amersham). Densitometric analysis of the protein bands was performed with the software Gel-Pro Analyzer (Media Cybernetics).

Apoptosis Detection and DNA Fragmentation Analysis. CaCo-2 cells without or with DHA treatment were stained with DAPI for nuclear staining and then scanned for characteristic changes in the nuclear material. This indicated convoluted budding and blebbing of the membrane, chromatin aggregation, and nuclear and cytoplasmic condensation pertaining to apoptosis. DNA fragmentation analysis was carried out using methods described earlier (26). Briefly, CaCo-2 cells without or with 48-h-DHA treatment were harvested by trypsinizing, and were suspended in 1-ml cell lysis buffer. The cell lysate was incubated at 55°C for 4–6 h. Cells were again treated with RNase (10 μg/ml) for 1 h at 37°C. The supernatant was collected, and DNA was extracted with phenol-chloroform. This procedure was repeated two or more times to obtain a clear aqueous phase that was then ethanol-precipitated and centrifuged. The pellet was then air dried and resuspended in 18 μl of distilled water. The final concentration of DNA was determined by UV absorbency at 260 nm. DNA (10 μg/lane) was electrophoresed on 1.8% agarose gels containing ethidium bromide (1 μg/ml). DHA-induced DNA fragmentation was confirmed by the appearance of internucleosomal cleavage, and the banding pattern as DNA ladder was photographed immediately.

RESULTS

Differential Gene Expression Pattern. Genes of which the expressions were altered >2-fold by DHA in CaCo-2 cells are shown in

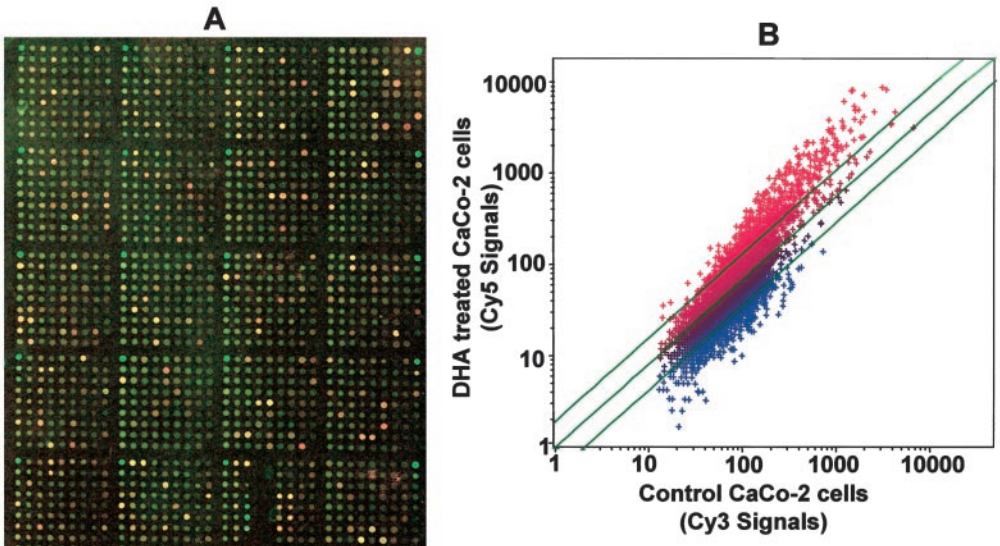


Fig. 2. *A*, scanned image of hybridized human oligoarrays containing 3.8 k genes. Total RNA from CaCo-2 cells, treated with DHA for 48 h, was used for microarray analysis as described in "Materials and Methods." A *red color image of spots* represents induced genes, *green spots* indicate repressed genes. *B*, scatter plot view of gene expression. Expression intensity Cy5:Cy3 ratios of untreated *versus* DHA-treated CaCo-2 cells. The ratios (Cy-5:Cy-3) of genes that have ≥2-fold expression are considered induced, and those with ≤0.5-fold expression are considered repressed. Approximately 504 of 3800 genes (13%) were expressed in DHA-treated cells.

Fig. 2*A* (scanned images from Atlas Glass Arrays with 3.8 k genes for human). The scatter graph (Fig. 2*B*) represents the gene expression patterns based on the magnitude of change in the intensity of Cy5/Cy3. To obtain an overall gene expression pattern on a specific cluster of genes in CaCo-2 cells treated with DHA, an in-depth analysis of microarray data was carried out. Results summarized in Table 1 indicate that DHA had a profound effect on various functional groups of genes, such as proinflammatory, cell cycle regulatory, and apoptosis-inducing genes, cGMP isoforms (phosphodiesterases), IFNs, alkaline phosphatases, and differentiation-inducing factors and genes. It is noteworthy that the functions of the genes in the expression profiles were diverse, including proinflammatory genes, caspases, pro- and antiapoptotic genes, cytokines, and IFNs. The changes in the levels of expression observed with the microarray analysis were confirmed with quantitative RT-PCR for a few selected genes using sequence-specific primers. In repeated experiments >85% of the expressed genes from microarray analyses could be confirmed by RT-PCR (Fig. 3). The data in Fig. 3 also confirms the expression of genes that are involved in the mechanism of inhibition of colon carcinogenesis by DHA. Although iNOS was not present in the array, we designed specific primers for human iNOS and performed RT-PCR by using the same RNA that was used for the microarray hybridization.

Cell Growth Inhibition and Induction of Apoptosis by DHA. In this study, we investigated the inhibitory effect of DHA on cell growth in exponentially growing CaCo-2 cells that were treated with DHA for 0, 24, and 48 h in the cell culture medium. Apoptotic cells, visualized by DAPI staining, indicated characteristic morphological changes, such as blebbing of the membrane, chromatin aggregation, and nuclear and cytoplasmic condensation pertaining to apoptosis (Fig. 4, *A* and *B*). Our results indicated that DHA inhibited cell growth by >54%, partly by inducing apoptosis (Fig. 4*C*). Internucleosomal cleavage of DNA, another indicator of apoptosis was confirmed by DNA fragmentation analysis. Degradation of chromosomal DNA into

Table 1 *Impact of DHA on the regulation of proinflammatory genes in CaCo2 cells*[a]

GenBank accession no.[b]	Coordinate[c]	Gene description	Mean ± SD[d]
Proinflammatory genes			
NM_000698	A2d9	Arachidonate 5-lipoxygenase-T1	0.27 ± 0.16
NM_000960	H2b1	Prostaglandin 12R	0.28 ± 0.13
NM_001139	A2d6	Arachidonate 12-lipoxygenase, R	0.43 ± 0.33
NM_000955	H2a7	Prostaglandin ER1	0.44 ± 0.11
NM_001629	A2e1	Arachidonate 5-lipoxygenase-AP	0.46 ± 0.09
NM_001141	A2d8	Arachidonate 15-lipoxygenase-T2	0.48 ± 0.15
NM_000959	H2a9	Prostaglandin F Receptor	0.48 ± 0.09
NM_000956	H2a8	Prostaglandin ER2	0.49 ± 0.16
NM_000954	K2i2	Prostaglandin D2s	0.59 ± 0.15
NM_000963	H2b2	Prostaglandin E2s	0.70 ± 0.17
		COX-2	0.76 ± 0.10
NM_000929	H1h9	Phospholipase A2, group V	0.77 ± 0.10
NM_000933	H1i1	Phospholipase C β 4	1.02 ± 0.03
NM_006506	I3f4	Ras p21 protein activator 2	1.34 ± 0.34
NM_005037	I2b7	PPAR γ	1.85 ± 0.28
NM_000697	A2d5	Arachidonate 12-lipoxygenase	1.89 ± 0.22
Cell cycle and apoptosis regulatory genes			
NM_003416	K4i8	Zinc finger protein 7	0.38 ± 0.13
NM_003420	L1a3	Zinc finger protein 35	0.41 ± 0.16
NM_003430	L1b1	Zinc finger protein 91	0.42 ± 0.17
NM_000964	H2b3	RXR α	0.44 ± 0.15
NM_005657	K1g6	p53-binding protein	0.45 ± 0.39
NM_003221	J3g9	AP-2 β (enhancer-binding protein)	0.45 ± 0.19
NM_000937	H1i3	Polymerase (RNA) II	0.47 ± 0.29
NM_003441	L1c1	Zinc finger protein 141	0.47 ± 0.20
NM_003806	L4a7	BCL2-interacting protein	0.48 ± 0.17
NM_000938	H1i4	Polymerase (RNA) II	0.50 ± 0.14
NM_006538	B2d1	BCL2-like 11 (apoptosis facilitator)	0.51 ± 0.18
NM_003419	L1a2	Zinc finger protein 10	0.54 ± 0.17
NM_003222	J3h1	AP-2 γ (enhancer-binding protein)	0.62 ± 0.20
NM_003217	J3g8	BAX inhibitor	1.02 ± 0.16
NM_002577	H2h6	p21-cdk inhibitor	2.01 ± 0.14
NM_001445	C3h3	Fatty acid binding protein T6	2.16 ± 0.13
NM_004375	C4d2	Cytochrome c oxidase	2.24 ± 0.07
NM_000134	B3f6	Fatty acid binding protein T2	2.44 ± 0.40
NM_002104	D3h3	Granzyme 3	3.02 ± 0.83
NM_001230	C2c4	Caspase 10	3.18 ± 0.86
NM_005256	D2a6	Growth arrest specific protein	3.84 ± 0.51
NM_002960	J1f6	S100 calcium binding protein A3	5.22 ± 0.81
cGMP isoforms			
NM_001083	K4a5	cGMP 5A	0.37 ± 0.22
NM_006204	I3a5	cGMP 6C	0.52 ± 0.45
NM_002601	H3a4	cGMP 6D	1.67 ± 0.19
NM_002602	H3a5	cGMP 6G	0.51 ± 0.37
NM_006205	I3a6	cGMP 6H	0.67 ± 0.54
NM_006259	I3e1	cGMP-dep.PK, type II	0.68 ± 0.44
Interferons			
NM_001571	F3b7	IFN regulatory factor 3	1.53 ± 0.26
NM_001550	F3a2	IFN related developmental regulator 1	1.47 ± 0.35
NM_004029	G1g7	IFN regulatory factor 7	1.45 ± 0.18
NM_001562	F3a9	Interleukin 18 (IFN-γ-inducing factor)	1.15 ± 0.38
NM_001548	F2i7	IFN induced protein with tetratricopeptide repeats 1	1.09 ± 0.21
NM_002462	G1e8	Homolog of murine (IFN-inducible protein p78)	1.07 ± 0.22
NM_005532	G2i3	IFN,α-inducible protein 27	1.02 ± 0.22
NM_001549	F2i8	IFN-induced protein with tetratricopeptide repeats 4	1.00 ± 0.20
NM_004705	I1g9	Protein-kinase, IFN-inducible double-stranded RNA dependent inhibitor, (P58 repressor)	0.99 ± 0.20
NM_002169	G4e8	IFN, α 5	0.98 ± 0.11
NM_002171	G4f1	IFN, α 10	0.85 ± 0.12
NM_005531	G2i2	IFN, γ-inducible protein 16	0.83 ± 0.15
NM_002176	F3f2	IFN, β 1, fibroblast	0.81 ± 0.16
NM_004120	D2g3	Guanylate binding protein 2, IFN-inducible	0.76 ± 0.16
NM_002175	G4f4	IFN, α 21	0.71 ± 0.10
NM_004510	G1i6	IFN-induced protein 75, 52kD	0.67 ± 0.18
NM_002177	F3f3	IFN ω 1	0.64 ± 0.20
NM_002053	D2h5	Guanylate binding protein 1, IFN-inducible, 67kD	0.61 ± 0.09
NM_002201	F3g2	IFN stimulated gene (20kD)	0.61 ± 0.10
NM_002173	G4f3	IFN, α 16	0.56 ± 0.13
NM_002172	G4f2	IFN, α 14	0.54 ± 0.05
NM_004509	G1i5	IFN-induced protein 41, 30kD	0.51 ± 0.18
NM_006900	G4h9	IFN, α 13	0.36 ± 0.05
NM_003690	L3b1	Protein kinase, IFN-inducible double-stranded RNA dependent activator	0.25 ± 0.05
Alkaline phosphatase and differentiation factors			
NM_005811	B2h5	Growth differentiation factor 11	0.73 ± 0.25
NM_001631	A2e2	Alkaline phosphatase, intestinal	0.74 ± 0.20
NM_001122	A1f8	Adipose differentiation-related protein	0.78 ± 0.15
NM_000557	K3i6	Growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	0.79 ± 0.36
NM_000478	A2e3	Alkaline phosphatase, liver/bone/kidney	0.86 ± 0.53
NM_001401	C3e3	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	0.88 ± 0.19
NM_005260	E4e6	Growth differentiation factor 9	0.94 ± 0.20
NM_004962	D4e7	Growth differentiation factor 10	1.36 ± 0.20
NM_005259	D1d7	Growth differentiation factor 8	1.51 ± 0.36
NM_001632	A2e4	Alkaline phosphatase, placental (Regan isozyme)	3.38 ± 1.13

[a] Overall gene expression profiled representing only selected functional groups of genes (out of 3800).

[b] GeneBank accession no. denotes Gene ID number for Gene Bank.

[c] Coordinate represents the position of the specific gene on the array for identical or homologous genes (Atlas Glass Array 3.8k human).

[d] Mean ± SD represent the cy3:cy5 ratios derived from microarray scanned images for DHA-treated *versus* untreated samples from three independent microarray data sets. Values are shown as fold up-regulation and down-regulation.

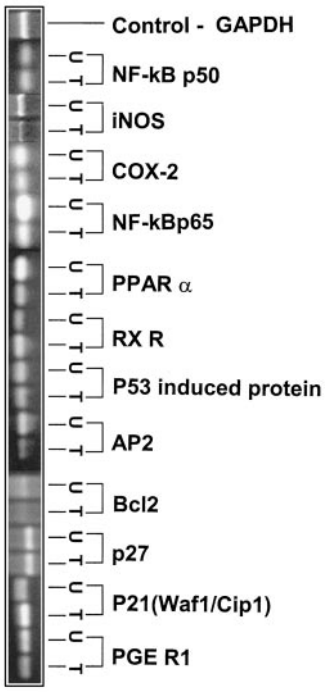


Fig. 3. RT-PCR validation of selected genes listed in Table 1. Differential expression of potential molecular targets modulated by DHA in CaCo-2 cells is shown on 2% agarose gel.

small oligo-nucleosomal fragments was clearly observed in cells treated with DHA after 24 and 48 h suggesting that DHA induces apoptosis in a time-dependent manner (Fig. 4*D*).

Down-Regulation of iNOS Expression. Immunofluorescence detection based on nuclear positive staining for iNOS expression demonstrated that the number of cells that were positive for iNOS were lower in CaCo-2 cells treated with DHA for 48 h than in the controls (Fig. 5, *A–D*). Importantly, the lower number of iNOS-positive cells observed in this study is consistent with an increase in the number of apoptotic cells as determined by DAPI staining. To relate the level of iNOS expression to the number of apoptotic cells, iNOS-positive cells and apoptotic cells were quantified. As shown in Fig. 6, iNOS expression was decreased in a dose-dependent manner by 2.5 and 5 μg of DHA, whereas apoptosis was enhanced by 2.5 μg DHA, but no additional enhancement with 5 μg of DHA was observed. Notably, the results from Western blot analysis indicate a dose-dependent inhibition of iNOS expression by DHA (Fig. 7*A*). Quantification by densitometry analysis of protein bands indicated a 2-fold down-regulation of the iNOS expression by DHA (Fig. 7*B*). However, a cause and effect relationship between the expression of iNOS and inhibition of apoptosis cannot be drawn from our results.

Down-Regulation of cGMP in DHA-treated CaCo-2 Cells. To determine whether there is any relationship between expression of cGMPs and other DHA-induced genes, a complete in-depth analysis was carried out to assess the expression of several isoforms of cGMP. Interestingly, we identified five clones of cGMP isoforms that were down-regulated by DHA (Fig. 8); however, the functional significance of these isoforms has yet to be determined with respect to iNOS inhibition, and activation of differentiation-inducing genes and related factors that are differentially expressed by DHA (Table 1).

Effect of DHA on Genes Involved in Differentiation. DNA microarray and RT-PCR analysis demonstrated activation of cyclin kinase inhibitor $p21^{(Waf1/Cip1)}$ and RXR at the mRNA level as shown in Fig. 3. The RXR is a nuclear receptor that functions as a ligand-activated transcription factor that selectively regulates cell differentiation and proliferation, making these ligands an ideal target for chemoprevention (28). Results from functional analysis of genomics from microarray data consistently indicated activation of several genes involved in cellular differentiation (Table 1; Fig. 9). Our data demonstrate that differential expression of genes involved in differentiation constituted ~0.1% of the total expressed genes. Specifically, up-regulation of alkaline phosphatase P and growth differentiation factors 8 and 10 indicates a potential role for DHA in colonic cell differentiation.

Effect of DHA on IFNs and NFκB. A major inhibitory effect of DHA was observed on expression of on IFNs α 5, 10, 21, 16, 14, 13, and γ and β isoforms. However, 25% of the IFNs did not show any remarkable changes (either activation or inhibition) as summarized in Table 1. RT-PCR analyses indicated a 2-fold inactivation of NFκB p65, although not much change could be observed in the transcripts of NFκB p50 in DHA-treated CaCo-2 cells. Repeated experiments using RT-PCR analyses revealed inactivation of iNOS and NFκB p65. It is noteworthy that major changes associated with CaCo-2 cell growth inhibition, inactivation of iNOS, and induction of apoptosis by DHA are consistent with the down-regulation of COX-2, NFκB p65 (Fig. 3), the family of IFNs, and several lipoxygenases as shown in Table 1. Additional studies are in progress to demonstrate the biological significance of differential expression patterns of iNOS, NFκB, and tumor necrosis factor isoforms by DHA.

DISCUSSION

The present study is part of a large-scale investigation on the chemopreventive efficacy of omega-3 PUFAs present in fish oil against colon carcinogenesis. This study was aimed at identifying signaling pathways that regulate colon cancer growth, development, differentiation, and apoptosis. Preclinical studies clearly demonstrate that diets rich in omega-3 PUFAs, including DHA, induce apoptosis, and inhibit COX-2 and iNOS activity in colon tumors (7). Identification of a subset of genes that are modulated by omega-3 PUFAs, including DHA, provides biomarkers for diet intervention studies in humans.

The outcome of this study is of great interest because of its implication for human colon cancer prevention. Earlier, we have demonstrated that DHA inhibits several proinflammatory genes, such as *COX-2*, and the prostaglandin family of genes in CaCo-2 colon cancer cells (15). The results of the present study clearly demonstrate for the first time that DHA inhibits iNOS expression and expression of associated genes in colon cancer cells. Because iNOS/NO and COX-2/prostaglandins appear to be involved in the pathogenesis of colon cancer (7, 14, 16, 29–32), selective inhibitors of these genes are likely chemopreventive agents. Indeed, our data support the concept that inhibitors of one or both of these inducible enzymes and their target genes are effective chemopreventive agents against colon carcinogenesis in preclinical models (7, 29–31).

Pathophysiological actions are induced by various forms of NO synthase that are mediated not only by free radical oxidants but also by activation of guanylate cyclase, leading to the production of cGMP. It is known that NO or its oxidation product, peroxynitrite, may activate COX-2 activity (33). As discussed earlier, only iNOS produces sustained NO concentrations in the micromolar range, and this inducible form is associated specifically with neoplastic tissue. In addition, NO has been found to post-translationally modify a number of important cellular proteins, including p53, caspases, and DNA repair enzymes (25, 34). Inactivation of iNOS and cyclic GMP by DHA suggests a strong protective mechanism that can abrogate any pathological effects induced by iNOS and cyclic GMP. However, a defined functional mechanism of DHA with respect to cyclic GMP

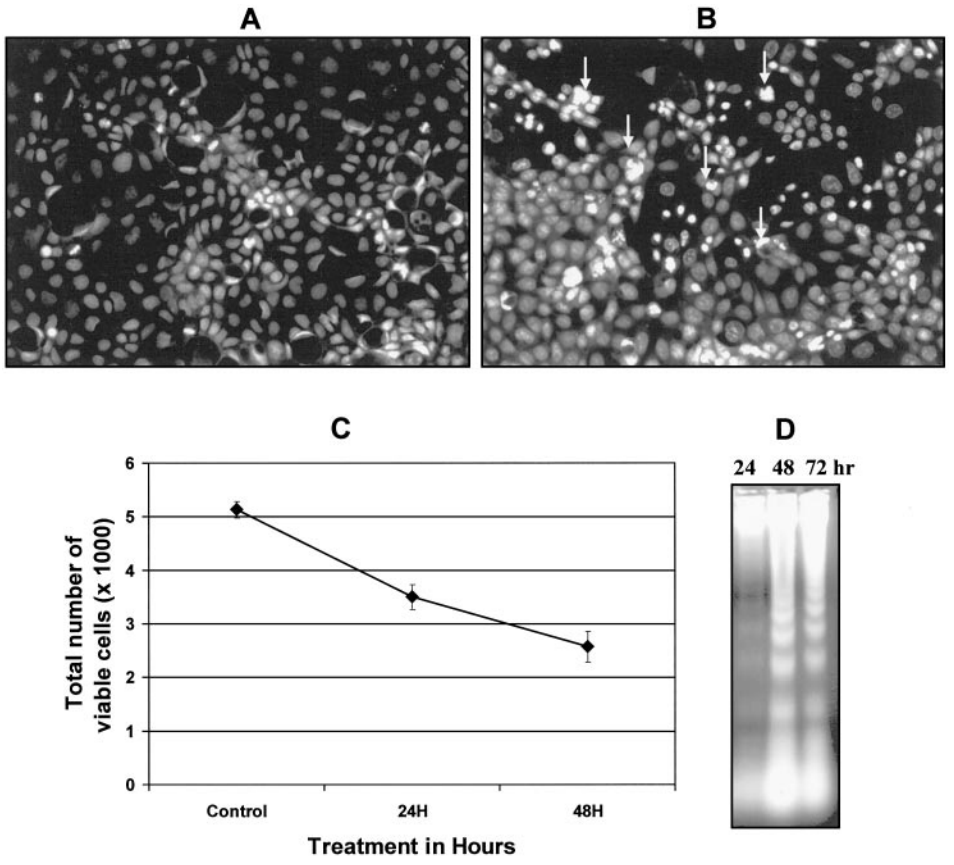


Fig. 4. Effect of DHA on inhibition of CaCo-2 cell growth and induction of apoptosis. *A*, untreated CaCo2 cells. *B*, DHA treated CaCo-2 cells, showing apoptotic cells (*arrow*) after 48 h. *C*, DHA effect on cell growth. *D*, DNA fragmentation analysis (1.8% agarose gel) showing fragmented DNA at 24, 48, and 72 h time intervals

regulation in colon cancer has yet to be established. The present study also demonstrates an inhibitory effect of DHA on the family of IFNs (Table 1), suggesting its anti-inflammatory properties. IFNs (α and β forms) are implicated in autocrine and paracrine signals critical for induction of murine iNOS (35). Our findings on inactivation of iNOS, and activation of proapoptotic and differentiation-inducing genes are consistent with observations from related studies that indicate an important role for DHA in cellular differentiation and apoptosis (36, 37). A study by Kielar *et al.* (38) points to the possibility that several proinflammatory factors that activate iNOS could be inactivated by

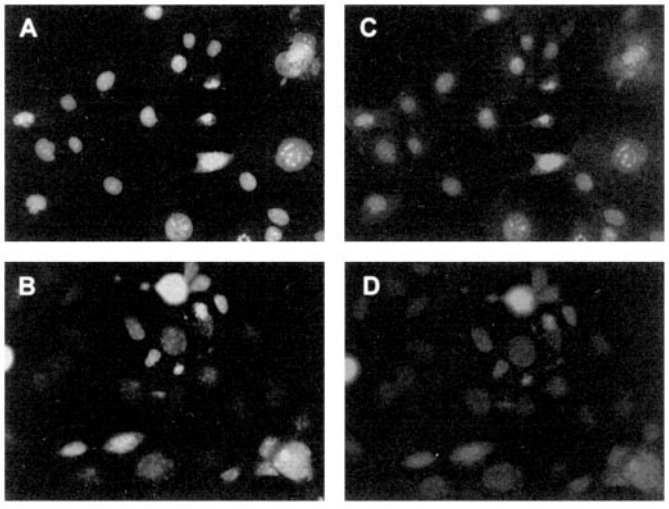


Fig. 5. Immunofluorescence detection of DHA effect on the expression of iNOS in CaCo-2 cells. *A*, untreated CaCo-2 cells (DAPI nuclear staining); *B*, DHA-treated CaCo-2 cells (DAPI nuclear staining); *C*, untreated CaCo-2 cells; *D*, DHA-treated CaCo-2 cells

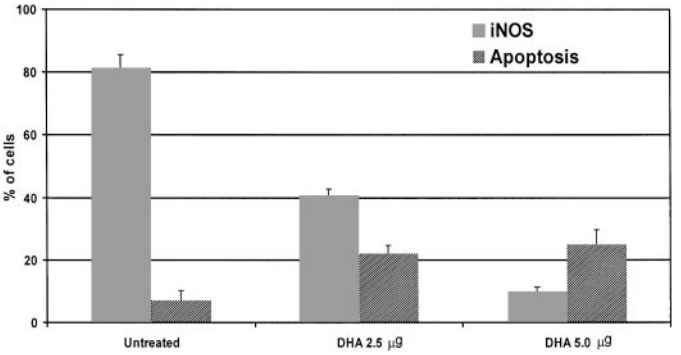


Fig. 6. Effect of DHA on iNOS-positive and apoptotic cells. Percentage of apoptotic cells was determined by DAPI staining. DAPI-positive cells with characteristic nuclear condensation and DNA strand breaks for apoptosis were counted from 10 identical fields using a fluorescence microscope (Olympus) with ×40 magnification; *bars*, ±SD.

DHA via down-regulation of NFκB and other target genes; however, this needs to be substantiated.

Our present study also determined whether DHA treatment influences the CaCO-2 cells to undergo differentiation associated with apoptosis as a function of colonic tissue homeostasis. A >2-fold activation of $p21^{(Waf\text{-}1/Cip1)}$, corresponding with a change in the expression of retinoic acid receptor RXRα, additionally supports our observation on DHA-induced differentiation in colonic epithelial cells.

On the basis of our results, we propose that the mechanism(s) involved in the suppression of colon carcinogenesis by DHA are more likely multiple in nature as shown in Fig. 10. It is very clear that DHA inhibits the iNOS expression at the mRNA and protein levels by reprogramming the expression of several proinflammatory genes that, in turn, might have induced a negative effect on the transcription of

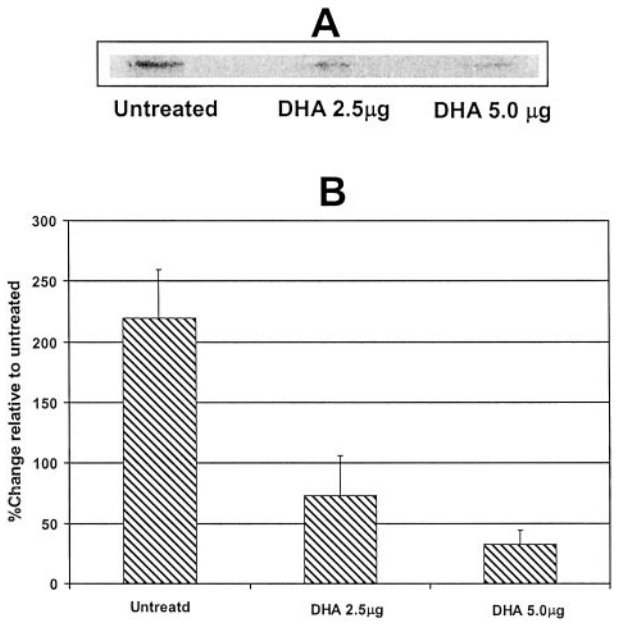


Fig. 7. Effect of DHA on iNOS expression. *A*, Western blot analysis of CaCo-2 cell lysate for iNOS expression after treatment with DHA (2.5 μg/ml and 5.0 μg/ml, respectively) for 48 h. *B*, densitometric analysis of iNOS protein bands as altered by DHA; *bars*, ±SD.

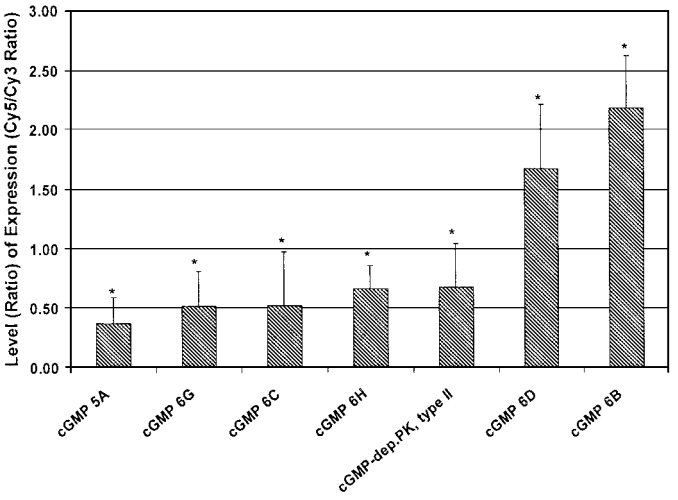


Fig. 8. Differential expression of cGMP isoforms. Differential expression is shown as the ratio between DHA-treated *versus* untreated CaCo-2 cells. More than 2-fold expression is considered up-regulated. cGMP isoforms include: cGMP 5A phosphodiesterase, cGMP 6G phosphodiesterase, cGMP 6C phosphodiesterase, cGMP 6H phosphodiesterase, cGMP-dep.PK type II, cGMP 6D phosphodiesterase, and cGMP 6B phosphodiesterase; *bars*, ±SD.

nuclear transcription factor NFκB and IFNs. It is also evident that DHA induces colonic cell differentiation partly through the inhibition of iNOS, and at the same time, by activating cyclin-dependent kinase inhibitor p21, known for its role in mammalian cell differentiation. Importantly, the RXR functions as a ligand-activated transcription factor that modulates cell differentiation, making its ligand an ideal target for chemoprevention (28). Thus, DHA, which acts as an RXR agonist, is a promising, naturally occurring ligand for chemoprevention of colon carcinogenesis.

In summary, we report here for the first time a vast array of DHA-responsive signaling genes and molecules representing more than one signaling pathway involved in colon cancer growth inhibition. The modulation of colon cancer cell growth by DHA is apparently mediated through the inhibition of COX-2 and iNOS expressions, and induction of apoptosis. An inhibitory effect on differential expression of NFκB and tumor necrosis factor receptor isoforms observed in RT-PCR and microarray analysis also suggests a synergistic effect induced by DHA on iNOS regulation. NFκB, which regulates several genes that are involved in the inflammatory process, provides an excellent target for development of new chemopreventive agents such as DHA. The results of this and our earlier study (15) suggest that the molecular targets modulated by DHA may be suitable indicators of effective chemopreventive intervention by selective agents. Our continuing studies may help to identify molecular targets of chemoprevention in colon carcinogenesis. The ability of diet rich in omega-3 PUFAs, including DHA, to modulate several molecular parameters associated with colon carcinogenesis strengthens the concept that a combination of agents targeting various molecular parameters may effectively inhibit colon cancer progression in humans.

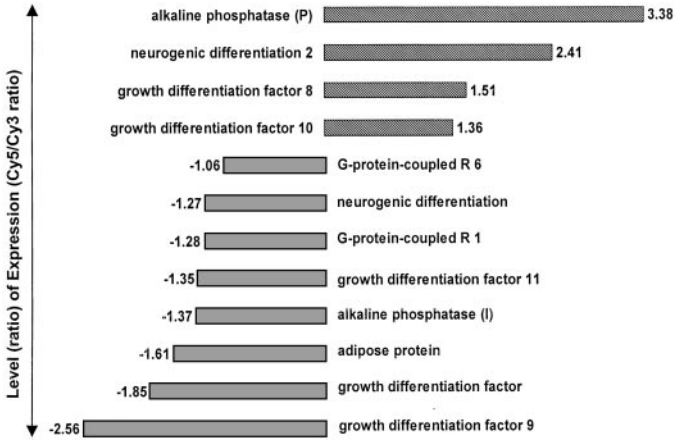


Fig. 9. Effect of DHA on levels of expression of genes related to differentiation. Functional analysis of genomics from microarray data demonstrated activation of several genes involved in cellular differentiation.

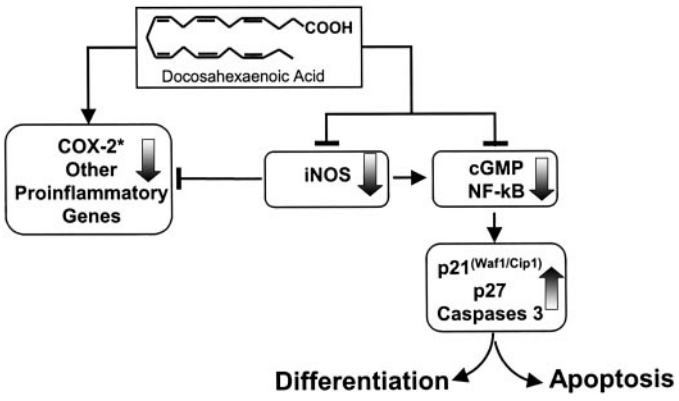


Fig. 10. Schematic diagram of potential molecular mechanisms of DHA. The illustration presented here depicts the key molecular and cellular events mediated by DHA in inhibiting COX-2 and iNOS target genes. Altered expressions of the above genes at the mRNA and protein levels in CaCo-2 cells after 48 h of DHA treatment were evident from the DNA microarray RT-PCR analysis and Western blot analysis. At the transcription level a simultaneous reprogramming of genes involved in differentiation, such as $p21^{(waf1/Cip1)}$, p27, and apoptosis by activating caspases (see Table 1) is evident from the present study. Because iNOS inhibition and p21 expression can be both p53-dependent and -independent pathways, there may be multiple pathways for the chemopreventive action of DHA. The cascade of molecular events regulated by DHA shows a unique relationship between proinflammatory genes, including COX-2, iNOS, and differentiation-initiating factors that result in the maintenance of colonic tissue homeostasis.

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