Omega-3 Polyunsaturated Fatty Acids Down-Modulate CXCR4 Expression and Function in MDA-MB-231 Breast Cancer Cells

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Abstract

Metastasis is the leading cause of death from breast cancer. A major factor of metastasis is the migration of cancerous cells to other tissues by way of up-regulated chemokine receptors, such as CXCR4, on the cell surface. Much is known of the beneficial effects of omega-3 polyunsaturated fatty acids (n-3 PUFA) on cancer; however, the mechanisms behind these effects are unclear. For this study, we investigated the effects of two n-3 PUFAs, docosahexaenoic acid and eicosapentaenoic acid, on CXCR4 expression and activity in the MDA-MB-231 breast cancer cell line. We compared the n-3 PUFAs with the saturated fatty acid stearic acid as a control. Treatment of the cells with n-3 PUFAs resulted in reduced surface expression of CXCR4, but had no effect on overall CXCR4 expression. Consequently, we found that the fatty acid treatment significantly reduced CXCR4-mediated cell migration. Successful CXCR4-mediated signaling and migration requires the cholesterol-rich membrane microdomains known as lipid rafts. Treatment with n-3 PUFAs disrupted the lipid raft domains in a manner similar to methyl-β-cyclodextrin and resulted in a partial displacement of CXCR4, suggesting a possible mechanism behind the reduced CXCR4 activity. These results were not observed in cells treated with stearic acid. Together, our data suggest that n-3 PUFAs may have a preventative effect on breast cancer metastasis in vitro. This suggests a previously unreported potential benefit of n-3 PUFAs to patients with metastatic breast cancer. The data presented in this study may also translate to other disorders that involve up-regulated chemokine receptors. (Mol Cancer Res 2009;7(7):1013–20)

Introduction

Metastasis is the leading cause of death from breast cancer. One of the hallmarks for breast cancer metastasis is the overexpression of chemokine receptors, which leads to migration of the cancerous cells to surrounding tissues (1). Chemokines are a subfamily of cytokines that carry out important functions such as cell survival, cell growth, cell migration, and cell differentiation (2, 3). Chemokines fall into four families that are denoted by the amino acid sequence at the conserved cysteines near the NH2 terminus. Chemokine receptors are named for the conserved sequence found in the corresponding ligands. Chemokines and chemokine receptors have been implicated heavily in different cancers, including breast cancer (4, 5). Overexpression of chemokine receptors or ligands can lead to enhanced survival, migration, and unchecked growth of the tumor cells (6). Some current therapies are designed to block the up-regulated chemokine receptors as a treatment for cancer and cancer metastasis (7, 8).

CXCR4 is a transmembrane G-protein–coupled receptor that is involved in numerous functions including lymphopoiesis, hematopoietic stem cell homing and engraftment, cell survival enhancement, and HIV viral entry (2). CXCR4 has only one known ligand, CXCL12/stromal cell–derived factor 1. CXCR4 is the most prominent chemokine receptor expressed on breast cancer cells and is not expressed on normal breast cells (1). CXCR4 is a major factor in breast cancer metastasis due to migration of the cancerous cells through CXCL12 signaling to surrounding tissues (9-14). This is achieved largely because CXCL12 is present in almost all major tissues. CXCR4 signaling requires receptor dimerization on ligand binding and is regulated in cholesterol-rich microdomains on the cell plasma membrane known as lipid rafts (15, 16). Disruption of the lipid rafts by methyl-β-cyclodextrin inhibits the migration of CXCR4+ cells to CXCL12.

Populations that subsist on diets consisting mostly of fish have been reported to have lower cancer incidence rates (17, 18). We and others have shown that omega-3 polyunsaturated fatty acids (n-3 PUFA), namely, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have demonstrable anticancer properties both in vitro and in vivo; however, the mechanisms behind the benefits are not clear (19-22). Incorporation of DHA and EPA into the plasma membrane leads to disruption of the lipid raft domains (23). One of the markers for lipid rafts, caveolin-1, is partially displaced on treatment with DHA and EPA (24, 25). Similarly, n-3 PUFAs have been shown to displace other signaling proteins from lipid rafts (26). Because CXCR4 is one protein that requires intact lipid rafts for signaling, we hypothesized that n-3 PUFA treatment would alter the surface expression and function of CXCR4 on the metastatic MDA-MB-231 breast cancer cell line.
During the present investigation, we found that exposure to n-3 PUFAs resulted in decreased surface levels of CXCR4 on the aggressive breast cancer cell line MDA-MB-231 in a dose- and time-dependent manner, whereas saturated stearic acid had no effect. Conversely, we found that overall CXCR4 expression was unaffected by the fatty acids, suggesting a membrane alteration as the mechanism behind the reduced surface expression. We observed that migration of the MDA-MB-231 cells toward the CXCR4 ligand CXCL12 was significantly reduced on n-3 PUFA treatment. Our data suggest that the disruption of required lipid raft domains for CXCR4 signaling and the displacement of CXCR4 from the lipid raft domains are potential mechanisms behind the inhibited migratory response after DHA and EPA treatment.

**Results**

**Effects of n-3 PUFAs on CXCR4 Expression**

Expression of CXCR4 was qualified by flow cytometry and shown in Fig. 1A. We observed a significant decrease in surface CXCR4 expression in cells treated with 25 μmol/L DHA and EPA. In contrast, stearic acid did not affect CXCR4 expression. Potential cytotoxicity issues were ruled out by trypan exclusion (data not shown). We further performed the experiment with escalating doses of the fatty acids and a time course. The qualitative data presented in Fig. 1B to D are further evidence that n-3 PUFAs reduced CXCR4 surface expression in a dose-dependent manner. We observed that significant decrease occurred at all concentrations. The highest reduction in expression occurred at 24 hours, with a slight but statistically insignificant increase at 48 hours. This could be due to the metabolism of the fatty acids and suggests that the effect is reversible over time. DHA and EPA at concentrations as low as 12.5 μmol/L significantly decreased CXCR4 expression by ~25% at 24 hours (Fig. 1B). At the highest doses of 50 μmol/L of DHA and EPA, CXCR4 surface expression was reduced by ~75% (Fig. 1C). The difference in CXCR4 expression between cells treated with DHA and cells treated with EPA was not statistically significant.

**FIGURE 1.** Surface expression of CXCR4 is decreased after treatment with n-3 PUFAs. High CXCR4 expressing MDA-MB-231 cells (1 × 10⁶) were treated with escalating doses of DHA, EPA, stearic acid (SA), or mock control in serum-free medium for indicated times. Following treatment, CXCR4 surface expression was measured by flow cytometry. A, Representative histograms of cells treated with 25 μmol/L fatty acids at 24 h. Surface expression of CXCR4 was quantified and represented as the percent mean fluorescence intensity of an untreated sample. Cells were treated with 12.5 μmol/L fatty acid (B), 25 μmol/L fatty acid (C), and 50 μmol/L (D). Results are presented from three triplicate experiments.

**FIGURE 2.** Effect of n-3 PUFAs on total CXCR4 expression. A. High CXCR4 expressing MDA-MB-231 cells were treated with 25 μmol/L n-3 PUFAs or controls for 24 and 48 h. Following treatment, the cells were lysed and protein concentrations were measured using bicinchoninic acid reagent. SDS-PAGE was done. Lane 1, untreated control; lane 2, mock control; lane 3, DHA (25 μmol/L); lane 4, EPA (25 μmol/L); and lane 5, SA (25 μmol/L). Results are representative of two separate duplicate experiments. B. Proteins were quantified by densitometry. Columns, mean percent of the untreated control observed at the 24-h time point; bars, SD.
To analyze total CXCR4 expression, we used SDS-PAGE of lysates generated from cells treated with n-3 PUFAs and controls. This was done to determine if the reduced protein surface expression was the result of protein degradation or changes in translation. We found that overall CXCR4 protein expression was unaffected by the n-3 PUFA treatment at the time and concentration used in the flow cytometry experiments (Fig. 2A and B), suggesting that the reduced surface expression of CXCR4 was a result of membrane changes that rendered the protein inaccessible to the flow cytometry antibody and not due to protein degradation. Interestingly, we observed that the overall CXCR4 protein decreased in all treatments except stearic acid between 24 and 48 hours (Fig. 2A).

**Effects of n-3 PUFAs on CXCR4-Mediated Cell Migration**

CXCR4-mediated cell migration is a hallmark for breast cancer cell metastasis. Whereas the surface expression of CXCR4 was significantly reduced by n-3 PUFA treatment (Fig. 1A and B), it was not eliminated entirely. To investigate whether this reduction in surface expression had a functional consequence, we used an *in vitro* cell migration assay. Cells treated with 25 μmol/L n-3 PUFAs consistently displayed significantly reduced migration toward CXCL12, whereas cells treated with stearic acid or the mock control showed no reduction in migration (Fig. 3A and B). At 24 hours, migration in DHA-treated cells was significantly reduced by ~85% to levels of migration observed with the mock control, whereas migration in EPA-treated cells was significantly reduced by 75% (Fig. 3B). At 48 hours, we observed a slight increase in migration of cells treated with n-3 PUFAs. This result correlated with the slight increase in surface expression observed in Fig. 1. However, the migration of the n-3 PUFA–treated cells was still significantly lower compared with the mock and stearic acid controls at 48 hours.

**Effects of n-3 PUFAs on Lipid Raft Structure**

CXCR4 signaling on ligand activation requires receptor dimerization that occurs in cholesterol-rich lipid raft domains (15, 16). Previous studies report that n-3 PUFAs disrupt lipid rafts and alter the presence of signaling proteins in these rafts (23, 26). Cholera toxin B with the fluorescent tag Alexa Fluor 555 was used to stain the lipid rafts in MDA-MB-231 cells treated with n-3 PUFAs or controls. The cells were imaged and photographed. We observed that treatment of the cells with n-3 PUFAs resulted in a change of the lipid raft staining pattern (Fig. 4D and E) similar to those observed when cells were treated with methyl-β-cyclodextrin (Fig. 4F), a known disruptor of lipid rafts. This alteration was not observed in untreated cells or mock- and stearic acid–treated cells (Fig. 4A-C). This suggests that n-3 PUFAs disrupted the lipid rafts in a similar manner to methyl-β-cyclodextrin and further suggests a mechanism behind the down-regulated CXCR4 activity.

**Treatment with n-3 PUFAs Displaces CXCR4 in the Lipid Raft Domain**

We examined whether the disruption of the lipid raft domains by n-3 PUFAs could affect the localization of CXCR4. Detergent-insoluble lipid raft domains of cells treated with 25 μmol/L n-3 PUFAs or controls were isolated by sucrose gradient after treatment with DHA (Fig. 5C) and EPA (Fig. 5D) but not displaced by stearic acid (Fig. 5B) or mock (Fig. 5A) treatment. For cells that were treated with n-3 PUFAs, the CXCR4 was completely displaced from fraction 3 to fraction 4 (Fig. 5C and D). This was not observed in cells treated with stearic acid (Fig. 5B), suggesting that the partial displacement of CXCR4 from the lipid raft domain is a specific effect of n-3 PUFA treatment and a potential mechanism for the reduced CXCR4 surface expression and function.

**Discussion**

Chemokine signaling has stimulated interest in the cancer field for several reasons. Activities such as cell proliferation...
and migration are essential for tumor survival. Increased chemokine signaling through up-regulated receptors on tumor cell surfaces is a major factor in metastasis, a hallmark for late-stage cancer patients (11, 12, 14, 27). The reported levels of surface expression of CXCR4 on MDA-MB-231 cells have varied between different laboratories; however, the consensus of the various reports is that the CXCR4 is a major factor in cell migration and metastasis (9-14, 28). Here we report for the first time that n-3 PUFAs down-modulate CXCR4 molecules on the surfaces of the breast cancer cell line MDA-MD-231 and suppress CXCL12-mediated migration. These observations were made using both low CXCR4 expressing cell lines from American Type Culture Collection (data not shown) and high CXCR4 expressing cell lines derived from lung tumors (14) and provided by Dr. Nakshatri. These observations suggest that n-3 PUFAs may have beneficial effects on cancer metastasis. The focus of this article is on the CXCR4-CXCL12 axis. The similar structures and functions of the other chemokine receptors, coupled with the fact that other cell surface molecules such as endothelial growth factor receptor and interleukin-2 receptor are displaced by n-3 PUFA treatment (29, 30), suggest that other surface molecules that support migration may be affected in the same manner. This would be particularly true for molecules that require the lipid raft domains for signaling.

We hypothesize that n-3 PUFA treatment reduces CXCR4 signaling, and therefore cell migration, through more than one mechanism (Fig. 6). First, the decrease in surface expression of CXCR4 means that fewer agonist reactions take place, leading to lower signaling and reduced migration. The observation through Western blots that total CXCR4 expression is unchanged by n-3 PUFA treatment suggests that whereas the surface protein is reduced, it is not a function of protein degradation but rather that of protein sequestering or relocation. A second potential mechanism behind the reduced CXCR4 activity is the incorporation of n-3 PUFAs into the cell membrane and consequent disruption of cholesterol-rich lipid rafts (Fig. 4). Previous reports have shown that the lipid raft domains are required for CXCR4 to dimerize, signal through the nuclear factor-κB pathway, and eventually cause cell migration (15, 16). Our data confirm that treatment with n-3 PUFAs disrupts the lipid rafts, resulting in a cholera toxin B staining profile similar to the known lipid raft disruptor methyl-β-cyclodextrin (31-33). Future investigation will be required to determine whether the lipid raft disruption by n-3 PUFAs results in decreased receptor dimerization.

Caveolin-1 is a protein that is associated with lipid rafts (34, 35). Previous reports in the literature suggest that treatment of cells with DHA and EPA causes a partial displacement of caveolin-1 from the lipid rafts and decreases signaling from molecules such as interleukin-1 in human umbilical cord endothelial cells (24, 25). Our observations confirmed the previous literature and showed that the phenomenon also occurs in MDA-MB-231 breast cancer cells (Fig. 5). A sequence scan of CXCR4 identifies a caveolin-1 consensus binding site (ΦΨΦΨXXXΨ; YAFLGAKF) at position 310, the interface between the carboxyl tail and the seventh transmembrane domain (36, 37). We observed that treatment of MDA-MB-231 cells resulted in a partial displacement of CXCR4 similar to that of caveolin-1. We suggest a mechanism where CXCR4 binds to caveolin-1 through the consensus binding site. The partial displacement of caveolin-1 from the lipid raft domains brought about by n-3 PUFAs may pull the surface CXCR4 with it to cellular regions inaccessible to both the flow cytometry staining antibody and the ligand, CXCL12. Whether the CXCR4 was partially displaced through interaction with caveolin-1 or through a similar but independent mechanism from caveolin-1 will require further analysis. It should be noted that the consensus binding sequence is conserved in same region of most chemokine receptors. It is therefore possible that other chemokine receptors may also be affected by changes in lipid raft domains.

Whereas previous reports have suggested that n-3 PUFAs decrease the survival and proliferation of breast cancer cells, the extent of this decrease is unknown. Wu et al. (22) showed that free DHA and free EPA are cytotoxic to breast cancer cells in a dose-dependent manner. Conversely, we observed that the
concentrations of DHA, EPA, and stearic acid used for this study were not cytotoxic. This is potentially due to the fact that they used free DHA and EPA, compared with this study, which used bovine serum albumin (BSA)–conjugated fatty acids. This suggests that oxidation may play a role in the previously reported beneficial effects of DHA and EPA because albumin is known to have antioxidative effects (38). However, stearic acid is also known to be cytotoxic at certain concentrations (39) but had no effects on CXCR4 expression and cell migration (Figs. 1 and 3), suggesting that whereas a portion of the cancerous cells may be eliminated by the fatty acids, the remaining cells after stearic acid treatment are still capable of metastasizing.

The data presented in this study suggest that a diet rich in omega-3 fatty acids could have a beneficial effect for patients suffering from late-stage metastatic breast cancer. Further investigation will be required to determine whether the effects observed in the breast cancer cell lines will be replicated with primary breast cancer cells. Further investigation will also be required to see if the effects seen in vitro will translate in vivo. It will be important to determine the levels of omega-3 fatty acids needed to be consumed for the effect to be observed. Reduced expression and activity of CXCR4 from n-3 PUFAs has potential for breast cancer as well as other fields of study. Up-regulated surface receptors play a role in numerous diseases including, but not limited to, HIV (40), other cancers (41), atherosclerosis (42), and Alzheimer’s disease (43). The reduction of surface expression and disruption of required lipid rafts could have beneficial effects for most of these diseases.

Materials and Methods

Cell Lines and Reagents

The breast cancer cell line MDA-MB-231 (American Type Culture Collection) was maintained in low-glucose DMEM (Invitrogen) containing 10% w/v fetal bovine serum (Invitrogen) and supplemented with penicillin and streptomycin (Invitrogen). High CXCR4 expressing MDA-MB-231 cells were a generous gift from Dr. Harikrishna Nakshatri (Indiana University School of Medicine, Indianapolis, IN) and were maintained in MEM supplemented with 10% (w/v) fetal bovine serum, 10 mmol/L insulin, and antibiotics as previously described (14). Western antibodies to CXCR4, caveolin-1, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology. Antibodies for flow cytometry were purchased from eBioscience. Fatty acids were purchased from Nu-chek Prep, Inc. WST-1 cell proliferation reagent was purchased from Roche. Recombinant CXCL12 was purchased from R&D Systems. Methyl-β-cyclodextrin was purchased from Sigma-Aldrich.

Fatty Acid Treatment

Stock solutions (1 mmol/L) of the polyunsaturated fatty acids and controls were prepared by complexing free fatty acids with fatty acid–free BSA to improve stability (44). MDA-MB-231 cells were treated with the fatty acids in serum-free medium supplemented with penicillin and streptomycin at concentrations and times denoted in the figure legends. Before the addition of the fatty acids, the cells were washed once with serum-free medium containing antibiotics.

Flow Cytometry

After treatment with the fatty acids and controls, the cells were harvested and washed in cold PBS containing 1% BSA. The cells were suspended in 1% BSA in PBS containing the phycoerythrin-conjugated 12G5 epitope monoclonal CXCR4 antibody or the isotype control antibody for 30 min. Cells were then washed consecutively with 1 mL of 1% BSA in PBS and 0.1% BSA in PBS. Analysis was done on a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser emitting at 488-nm wavelength.

FIGURE 5. Effects of n-3 PUFAs on membrane distribution of CXCR4. Low CXCR4 expressing MDA-MB-231 cells (20 × 10⁶) were treated with mock control (A), and 25 μmol/L stearic acid (B), DHA (C), and EPA (D) for 4 h. The cell lysates were subjected to discontinuous sucrose density gradient ultracentrifugation to separate lipid raft fractions as described in Materials and Methods. Fractionated samples were concentrated through methanol precipitation and analyzed by Western blotting for the presence of CXCR4 and caveolin-1. Representative of three separate experiments.
Fluorescence was detected through a 575 ± 26-nm band pass filter and quantified using CellQuest Software (Becton Dickinson). Quantification results are presented as percent mean fluorescence intensity of the untreated control in triplicate assays, using gated cell populations that exclude dead cells and cellular debris.

**Western Blot Analysis**

Protein extracts were prepared as whole-cell lysates in radioimmunoprecipitation assay buffer containing protease inhibitors (Roche). Lysates were assayed for protein concentration by bicinchoninic acid assay (Pierce) and subjected to SDS-PAGE. The resulting blots were probed for CXCR4, stripped, and reprobed for glyceraldehyde-3-phosphate dehydrogenase. Membranes were developed using a chemiluminescence detection kit from Amersham Biosciences.

**Cell Migration**

Cell migration assays were done as previously described (45) with some modification. Briefly, fatty acid–treated and untreated MDA-MB-231 cells were washed in chemotaxis medium (Iscove’s modified Dulbecco’s medium supplemented with 0.5% BSA). Cells (3 × 10⁶/mL) were placed in 8-μm Transwell chambers (Costar) at volumes of 100 μL. The inserts were placed into wells containing 600 μL chemotaxis medium with or without 100 ng/mL CXCL12. The plates were incubated for 4 h at 37°C and 5% CO₂. To halt migration, the inserts were removed from the upper compartment and the migrated cells that adhered to the bottom of the Transwell membrane were stained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/mL). Migration was quantified on an Olympus fluorescent microscope (model no. BX40) by enumerating the average cell number in three randomly selected fields of view (200×) on three separate filters (46). Chemo- tactic indexes were calculated by dividing the migrated cells for each treatment by the average number of migrated cells on membranes that had no CXCL12 in the bottom well. Images were recorded with a MicroFire digital camera with PictureFrame software (Optronics).

**Immunofluorescence**

MDA-MB-231 cells were grown and treated, as denoted in the figure legend, on chamber slides. After treatment, the cells were fixed in 3% paraformaldehyde. The cells were blocked in a PBS solution containing 2% BSA, 2% fetal bovine serum, and 0.1% NP40 for 30 min. Lipid raft staining was carried out with β-cholera toxin conjugated to Alexa Fluor 555 (Invitrogen). After three washes with PBS containing 0.1% NP40, the cell nuclei were stained with DAPI and imaged on a Leica inverted fluorescent microscope (model no. DMI4000B). Images were recorded and merged with Spot Advanced software (Diagnostic Instruments).

**Lipid Raft Isolation**

Assays were done as previously described with some modification (23). Briefly, MDA-MB-231 cells were washed with 10 mL serum-free DMEM and then treated with 25 μmol/L fatty acids under serum-free conditions. The flasks were incubated at 37°C and 5% CO₂ for 4 h. The treatments were terminated by aspirating the flask contents and washing the cells with 10 mL cold MES-buffered saline (150 mmol/L NaCl, 25 mmol/L MES, pH 6.5). The cells were then scraped and centrifuged at 800 × g for 5 min. The cell pellets were mixed with 1-mL cold lysis buffer [MES-buffered saline, 1% Triton, protease inhibitors...
(Roche)] and incubated at 4°C for 30 min. After the incubations, the cells were homogenized on ice using a Dounce homogenizer and diluted to 2.5 mL in lysis buffer. The lysates were adjusted to include 45% sucrose at a volume of 4 mL and subjected to a discontinuous sucrose gradient containing 5%, 35%, and 45% sucrose. The sucrose gradients were ultracentrifuged at 200,000 × g, 4°C for 20 h. Following centrifugation, 1-mL fractions were collected from the top of each gradient and transferred to Eppendorf tubes. An aliquot of 100 μL from each fraction was used to precipitate the proteins using methanol as previously described (47). The concentrated proteins were reconstituted in loading buffer and subjected to SDS-PAGE. The resulting blots were probed for CXCR4, stripped, and reprobed for caveolin-1.

Statistics
All experiments were done at least thrice each in triplicate and expressed as mean ± SE. Comparisons were done using a Student’s t test. Significance was defined as P < 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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