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An omega-3 polyunsaturated fatty acid derivative, 18-HEPE, protects against CXCR4-associated melanoma metastasis

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Abstract

Melanoma has a high propensity to metastasize and exhibits a poor response to classical therapies. Dysregulation of the chemokine receptor gene CXCR4 is associated with melanoma progression, and although n-3 polyunsaturated fatty acids (PUFAs) are known to be beneficial for melanoma prevention, the underlying mechanism of this effect is unclear. Here, we used the n-3 fatty acid desaturase (*Fat-1*) transgenic mouse model of endogenous n-3 PUFA synthesis to investigate the influence of elevated n-3 PUFA levels in a mouse model of metastatic melanoma. We found that relative to wild-type (WT) mice, *Fat-1* mice exhibited fewer pulmonary metastatic colonies and improved inflammatory indices, including reduced serum tumor necrosis factor alpha (TNF- α) levels and pulmonary myeloperoxidase activity. Differential PUFA metabolites in serum were considered a key factor to alter cancer cell travelling to lung, and we found that n-6 PUFAs such as arachidonic acid induced CXCR4 protein expression although n-3 PUFAs such as eicosapentaenoic acid (EPA) decreased CXCR4 levels. In addition, serum levels of the bioactive EPA metabolite, 18-HEPE, were elevated in *Fat-1* mice relative to WT mice, and 18-HEPE suppressed CXCR4 expression in B16-F0 cells. Moreover, relative to controls, numbers of pulmonary metastatic colonies were reduced in WT mice receiving intravenous injections either of 18-HEPE or 18-HEPE-pretreated melanoma cells. Our results indicate that 18-HEPE is a potential anticancer metabolite that mediates, at least in part, the preventive effect of n-3 PUFA on melanoma metastasis.

Introduction

One of the most common malignances in the USA is melanoma, the incidence of which among adults aged 50 and older has increased by 2.6% per year since 1960. Although melanoma accounts for <1% of all skin cancers, it contributes disproportionately to skin cancer deaths due to its rapid progression and high metastatic propensity (1,2). The cytotoxicity and ineffectiveness of conventional anticancer therapies such as chemotherapy and radiotherapy have posed a significant challenge to the treatment of metastatic melanoma (2,3).

Moreover, although more recent therapeutic advances such as immunotherapy and molecular-targeted therapy have improved prognosis and treatment for melanoma patients, problems such as individual genetic variation and drug resistance persist (2,4,5). A greater understanding of the molecular mechanisms of metastatic melanoma, as well as the impact of natural nutritional compounds on these processes, holds the promise of developing non-toxic treatments for melanoma patients.

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Abbreviations

AA	arachidonic acid
COX-2	cyclooxygenase-2
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GPR40	G-protein coupled receptor 40
MPO	myeloperoxidase
PGE ₂	prostaglandin E ₂
PUFA	polyunsaturated fatty acid
TGFβ	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
WT	wild-type.

Heterogeneity in melanocyte lineage, such as aberrant expression of microphthalmia-associated transcription factor, has been suggested to play a role in abnormal melanocyte development and to contribute to melanoma formation (6). In addition, the interplay between pro-inflammatory signaling and melanoma plasticity is considered to be a key determinant of metastatic progression (4). In general, inflammation promotes the release of cytokines and chemokines, resulting in activation of the MAP kinase (MAPK) pathway and downstream signaling nodes such as microphthalmia-associated transcription factor. These molecular events trigger the initiation of a reciprocally supportive signaling network between cancer cells and immune cells such as neutrophils, leading to the establishment of a microenvironment favorable to tumor development (7–10). The G-protein-coupled chemokine receptor, CXCR4, is expressed in a variety of immune cells and cancer tissues and is activated by its physiological ligand stromal cell-derived factor-1 (CXCL12). The CXCR4–CXCL12 axis interfaces with tumor necrosis factor alpha (TNF-α) signaling to activate Akt/MAPK pathways and promote inflammatory cancer cell proliferation, cell adhesion, angiogenesis and metastasis (11–17). Recent studies have demonstrated that CXCR4 antagonists sensitize tumor cells to chemotherapy and allow for non-invasive biomarker imaging to monitor cancer progression (18). Despite this promise, challenges such as side effects and multiple off-target effects remain in CXCR4-targeted cancer therapy, and the development of suitable combination treatments is necessary (18,19).

An antagonistic relationship exists between n-6 polyunsaturated fatty acids (n-6 PUFAs, rich in corn and sunflower oil), which promote inflammatory conditions including obesity/diabetes, cardiovascular disease and cancer, and n-3 polyunsaturated fatty acids (n-3 PUFAs, rich in fish oil), which are known to protect against these conditions (20,21). Indeed, the elevated dietary ratio of n-6:n-3 PUFAs (10–20:1) prevalent in Western societies is thought to contribute to raised risks for chronic inflammatory disease (20,22). On a mechanistic level, this antagonism is thought to involve the competitive inhibition by n-3 PUFAs, particularly eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA, C22:6), of cyclooxygenase-2 (COX-2), reducing the production of eicosanoids from the COX-2 substrate and n-6 PUFA, arachidonic acid (AA; C20:4). One of the products of COX-2 is prostaglandin E₂ (PGE₂), a pro-inflammatory lipid mediator that stimulates cytokine release to support the establishment of tumor-favorable microenvironment and the development of an aggressive tumor phenotype (23,24).

Preclinical *in vitro* and *in vivo* research has demonstrated that the n-3 PUFAs, EPA and DHA inhibit melanoma development (25) and that this inhibition involves a variety of distinct mechanisms. These include regulation of pRb phosphorylation status (26), enhanced apoptosis (27), potential downregulation of G-protein coupled receptor 40 (GPR40) (28), inhibition of

COX-2 activity and the production of PGE₂ and pro-inflammatory cytokines (29,30), a decrease of total β-catenin expression (31) and suppression of cell proliferation through oxidative DNA damage (32). Moreover, n-3 PUFA has been demonstrated to suppress metastasis in part by inhibiting expression of matrix metalloproteinase 9 (33), CXCR4 (34) and COX-2 (29). Although these data indicate that n-3 PUFAs are potential antitumor nutrients, laboratory-based studies are inconclusive and poorly defined (35,36), and there is a dearth of appropriately designed human studies to support the protective effect of n-3 PUFAs on melanoma metastasis (22). Here, we investigated the protective role of n-3 PUFAs in an animal model of melanoma metastasis.

Materials and methods

Cell culture

Murine B16-F0 cells were directly purchased from ATCC in 2014 (Manassas, VA; catalog number: CRL-6322), cells were expanded, aliquots were frozen and cells were used within 6 months of receipt or resuscitation. Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Grand Island, NY) as well as penicillin/streptomycin (100 U/ml; Sigma-Aldrich) were used in cell culture at 37°C, under 5% CO₂. To determine the effects of n-6 PUFAs and n-3 PUFAs or their metabolites including 18-HEPE (Cayman, Ann Arbor, MI) on CXCR4 expression, cells were treated with the indicated lipid molecules for 48 h and harvested for further analysis.

Animals and treatment

Fat-1 C57BL/6 transgenic mice were generated as described in a previous study (37), and wild-type (WT) mice were used as control. Mice were fed 10% corn oil diet and maintained under 12 h light/dark cycles at constant temperature 22°C with free access to food and water. To initiate lung metastasis, mice were intravenously injected with 5 × 10⁵ B16-F0 cells in 100 μl of phosphate-buffered saline and the pulmonary tumor colonies were examined after 3 weeks. WT female 8-week-old C57BL/6J mice were purchased from Charles River Laboratory (Wilmington, MA) and used to study the potential preventative effect of 18-HEPE on metastasis. In 18-HEPE-pretreated model, B16-F0 cells were treated with 400 pg/ml 18-HEPE and ethanol as control for 48 h, respectively. Prior to intravenous (i.v.) injection to the mice, the cell viability in the presence or absence of 18-HEPE was not different between two groups, and the detail information was described in the [Supplementary Materials](#), available at [Carcinogenesis Online](#). The tumor colonies were examined after 3 weeks. In 18-HEPE intervention model, 100 pg and 1 μg of 18-HEPE were given to each mouse through i.v. and intraperitoneal (i.p.) injection every other day, respectively. The control group was given with the same volume and carrier solution as 18-HEPE group received, which was 5% ethanol in 100 μl of phosphate-buffered saline via i.p. injection and 0.001% ethanol in 50 μl of phosphate-buffered saline via i.v. injection. The mice were killed after 3 weeks, and tumor colonies were compared between treatment group and the control group. Animal studies were approved by Institutional Animal Care and Animal Ethics Committee of Massachusetts General Hospital.

Systemic inflammation measurement

Concentrations of TNF-α and IL-17 were measured in 40 μl of plasma using a multiplex immunoassay kit (Bio-Plex Pro™ Mouse Cytokine Assay, Bio-Rad, Hercules, CA) and Luminex technology (Bio-Plex, Bio-Rad) following the manufacturer's protocol.

Lipid profile analysis

Fifty milligrams of lung were homogenized under liquid nitrogen, and the lipid was extracted using 2:1 chloroform and methanol at 4°C overnight. The lipid fraction was further methylated by adding 1:1 of hexane and 14% boron trifluoride/methanol and heated at 100°C for an hour. Fatty acid methyl esters were analyzed using an automated 6890N Network Gas Chromatography equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). Individual fatty acid was determined by retention time compared with a reference standard GLC461 (NuChek Prep., Elysian, MN).

Mediator lipidomics

Serum was collected from paired WT and Fat-1 transgenic mice and extracted using Bligh and Dyer method with minor modification as described previously (43). For quantification of fatty acid metabolites on these lipid samples, a liquid chromatography-tandem mass spectrometry-based mediator lipidomics analysis was performed using an ultra-high performance lipid chromatography (UPLC) systems (Waters) with a linear ion-trap quadrupole mass spectrometer (QTRAP5500; AB SCIEX) equipped with Acquity UPLC BEN C18 column (Waters) as mentioned (43). Tandem mass spectrometry analyses were conducted in negative-ion mode, and fatty acid metabolites were identified and quantified by multiple reaction monitoring.

Gene expression measurement

Total RNA was extracted from cells/tissues using Trizol reagent (Invitrogen). cDNA was synthesized from 1.5 µg of RNA using iScript™ Reverse Transcription Supermix (Bio-Rad). qPCR was performed using cDNA in an Mx3000P qPCR system (Stratagene, Agilent Technologies, Santa Clara, CA) with specific primers and iTaq™ Universal SYBR Green Supermix (Stratagene). Glyceraldehyde 3-phosphate dehydrogenase was used as internal standard control, and the relative abundance of expression was calculated by the $2^{-\Delta\Delta C_t}$ method. All procedures followed the manufacturer's instruction, and the primer sequence was listed as follows: CXCR4 forward 5'-TGC AGC AGG TAG CAG TGA AA-3' and reverse 5'-TGT ATA TAC TCA CAC TGA TCG GTT C-3'; transforming growth factor beta (TGFB) forward 5'-GAC TCT CCA CCT GCA AGA CC-3' and reverse 5'-GGA CTG GCG AGC CTT AGT TT-3'; GAPDH forward 5'-CGA CTT CAA CAG CAA CTC CCA CTC TTC C-3' and reverse 5'-TGG GTG GTC CAG GGT TTC TTA CTC CTT-3'.

Imaging analysis of MPO activity

Myeloperoxidase (MPO) activity was measured according to a method described in a previous study (40). Briefly, mice were intravenously injected with 137.5 µl of luminol-R including 125 µl of 40 mg/ml luminol (Sigma-Aldrich) and 12.5 µl of 100 pmol QD 800 (Life technology, Grand Island, NY) after 2 weeks of initiating lung metastasis. Then, mice were imaged in an IVIS Spectrum instrument (Caliper, Perkinelmer, Waltham, MA) for 5 min with an open filter.

Western blotting

Protein extracts were prepared in radio immunoprecipitation assay buffer (G-Biosciences, St. Louis, MO) which contains phosphatase (Sigma-Aldrich) and protease inhibitor cocktail (ThermoFisher Scientific, Waltham, MO) and collected after centrifugation. Protein concentration was determined by the Coomassie protein assay (ThermoFisher Scientific). Protein samples were then resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with CXCR4 antibody overnight and then with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). β -Actin or GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control in western blotting. Horseradish peroxidase activity was measured using Supersignal™ West Pico Chemiluminescent Substrate kit (ThermoFisher Scientific).

Immunohistochemistry (IHC) staining

Mouse lung tissues were fixed with 4% formalin and embedded with paraffin. Sections were stained with hematoxylin and eosin to assess the histological features. All sections were visualized under bright-field microscopy.

Statistical analysis

Data were shown as mean values $\pm$ SEM. The results were performed by Prism 5.0 (GraphPad) and RStudio softwares. Two comparisons were analyzed using the two-tailed Student's *t*-test, and multiple comparisons were performed using analysis of variance with least significance difference test. *P* value <0.05 was considered statistical significance.

Results

Inverse correlation between lung tissue n-3 PUFA levels and frequency of melanoma pulmonary metastasis

We set out to establish a mouse model that would allow us to evaluate the effect of the n-6:n-3 PUFA ratio on the frequency of melanoma pulmonary metastasis. To avoid dietary confounding factors, we utilized the Fat-1 transgenic mouse model, in which overexpression of *Caenorhabditis elegans* n-3 desaturase converts endogenous n-6 PUFAs to n-3 PUFAs, resulting in elevated tissue levels of n-3 PUFAs compared with WT mice (37). To induce pulmonary metastatic melanoma, Fat-1 and WT mice were intravenously injected with murine B16-F0 melanoma cells (38). Relative to WT mice, Fat-1 mice had fewer pulmonary tumor foci (Figure 1A) and smaller metastatic foci volume in both early and late stages of metastasis (Figure 1B). Next, to evaluate the association between tissue n-6:n-3 ratio and indices of melanoma metastasis, we used gas chromatography to analyze lung tissue fatty acid profiles 21 days after i.v. injection of B16-F0 cells. As shown in Table 1, lung tissue levels of n-3 PUFAs, including EPA, DPA and DHA, were higher in Fat-1 mice than in WT mice ($P < 0.05$), leading to a nearly 3-fold lower lung tissue n-6:n-3 fatty acid ratio in Fat-1 (3.0) than WT (8.4) mice ($P < 0.01$). These results indicated that n-3 PUFAs exerted an inhibitory effect on pulmonary metastasis of melanoma cells.

Metastasis-induced inflammation is reduced in Fat-1 transgenic mice

Since n-3 PUFAs have been shown to antagonize the pro-inflammatory effects of n-6 PUFAs (39), we hypothesized that the inhibitory effect of n-3 PUFAs on pulmonary melanoma metastasis might involve suppression of the inflammatory response in this organ. MPO is expressed in neutrophils and macrophages and is a well-characterized inflammatory marker. Using luminol-R-mediated chemiluminescence resonance energy transfer to measure MPO activity (40), we found that pulmonary MPO activity was higher in WT mice than in Fat-1 mice (Figure 2A). Consistent with this, serum levels of immune cell-expressed inflammatory factors with roles in tumor metastasis, such as IL-17 and TNF- α , were lower in Fat-1 mice than in WT mice ($P < 0.05$; Figure 2B). In further agreement, we found that expression levels in lung tissue of metastasis-related factors, including CXCR4 and TGFB, were reduced in Fat-1 mice relative to WT mice ($P < 0.05$; Figure 2C). Using IHC staining of lung tissue sections, we observed that CXCR4 production in B16-F0 cells in the pulmonary tumor colonies of WT mice was notably higher than in Fat-1 mice (Supplementary Figure S1A, available at Carcinogenesis Online). Overall, these results suggest that the mechanism by which n-3 PUFAs inhibit melanoma pulmonary metastasis involve suppression of pulmonary and systemic inflammation.

n-6 and n-3 PUFAs exhibit differential effect on CXCR4 expression

Since n-3 PUFAs have been demonstrated to suppress metastasis in part by inhibiting expression of CXCR4 (29,34), we hypothesized that the reduced levels of CXCR4 we observed in the pulmonary tumor tissues of Fat-1 mice might be due to an inhibitory effect of n-3 PUFAs or their metabolites on the expression of CXCR4 in melanoma cells. Consistent with this notion, we found that CXCR4 expression was significantly lower in B16-F0 cells cultured in the presence of Fat-1 serum than in B16-F0 cells cultured in the presence of WT mice serum

Figure 1. Inverse correlation between lung tissue n-3 PUFAs levels and frequency of melanoma pulmonary metastasis. Pulmonary metastasis was induced via i.v. injection of 5×10^5 B16-F0 cells, and the results were shown at the third week of initiation (21 days). (A) The representative image of pulmonary metastatic foci and the number of metastatic foci. (B) The immunohistochemical changes of lung. The values were presented as mean $\pm$ SEM ($n = 6-10$). * $P < 0.05$.

(Supplementary Figure S1B, available at *Carcinogenesis* Online). To determine the specific serum n-3 PUFA mediating this effect, we next compared the lipid profiles of WT and Fat-1 serum using liquid chromatography-tandem mass spectrometry. We found that levels of EPA and its derivatives 5-HEPE, 8-HEPE, 18-HEPE, 19-HETE, 17,18-diHETE and 17,18-EpETE were significantly higher in the serum of Fat-1 mice than in WT mice (Figure 3A–D), with a lower ratio of AA:EPA in Fat-1 mice compared with WT mice ($P < 0.001$; Figure 3E). We next incubated B16-F0 cells either with EPA or AA for 48 h and found that EPA suppresses CXCR4 expression although AA elicits an opposite effect on CXCR4 expression (Figure 4A). Since 18-HEPE has been reported as a potent anti-inflammatory lipid mediator (41,42), we further hypothesized that 18-HEPE might be the specific metabolite of n-3 PUFA that suppresses the expression of CXCR4 in B16-F0 cells. Consistent with this, incubation of B16-F0 cells with 18-HEPE at concentrations of 100 pg/ml or higher was sufficient to virtually abolish CXCR4 expression with no difference

Table 1. Profiles of n-6 and n-3 PUFAs in lung tissue from WT and Fat-1 transgenic mice after initiating melanoma metastasis

Lipid name ^a	WT	Fat-1
SFA	44.964 $\pm$ 1.596	44.845 $\pm$ 2.117
MUFA	24.361 $\pm$ 1.634	23.056 $\pm$ 1.959
PUFA	30.675 $\pm$ 0.232	32.099 $\pm$ 0.657
C18:2 n-6	12.798 $\pm$ 1.465	13.441 $\pm$ 1.187
C18:3 n-6	0.205 $\pm$ 0.017	0.184 $\pm$ 0.016
C18:3 n-3	0.085 $\pm$ 0.018	0.133 $\pm$ 0.022
C20:2 n-6	0.699 $\pm$ 0.029	0.683 $\pm$ 0.026
C20:3 n-6	1.645 $\pm$ 0.15	1.357 $\pm$ 0.106
C20:4 n-6	9.849 $\pm$ 1.065	7.091 $\pm$ 0.472
C20:5 n-3	0.89 $\pm$ 0.273	2.247 $\pm$ 0.391*
C22:2 n-6	0.174 $\pm$ 0.01	0.276 $\pm$ 0.094
C22:4 n-6	2.625 $\pm$ 0.288	1.715 $\pm$ 0.123*
C22:5 n-3	0.286 $\pm$ 0.034	1.351 $\pm$ 0.101***
C22:6 n-3	1.423 $\pm$ 0.132	3.621 $\pm$ 0.404**
n-6 PUFA	27.788 $\pm$ 0.225	24.563 $\pm$ 0.619**
n-3 PUFA	2.683 $\pm$ 0.288	7.352 $\pm$ 0.64***
n-6:n-3 ratio	10.369	3.341

Total lipids of lung tissues were extracted and subjected to analysis of gas chromatography. Ratio of n-6:n-3 fatty acids was calculated from (C18:2 n-6 + C20:2 n-6 + C20:3 n-6 + 20:4 n-6 + C22:2 n-6 + 22:4 n-6)/(C18:3 n-3 + C20:3 n-3 + 20:5 n-3 + 22:5 n-3 + 22:6 n-3). Some data of the fatty acid profile were not shown here, and the values (% of total fatty acids) were mean $\pm$ SEM ($n = 6$).

^aThe abbreviation was listed as follows: MUFA, monounsaturated fatty acid; SFA, saturated fatty acid. C18:2 n-6, linoleic acid; C18:3 n-6, γ -linolenic acid; C18:3 n-3, α -linolenic acid; C20:2 n-6, eicosadienoic acid; C20:3 n-6, dihomo- γ -linolenic acid; C20:4 n-6, arachidonic acid; C20:5 n-3, eicosapentaenoic acid; C22:2 n-6, docosadienoic acid; C22:4 n-6, adrenic acid; C22:5 n-3, docosapentaenoic acid; C22:6 n-3, docosahexaenoic acid.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in cell viability (Figure 4B and Supplemental Figure S2A). These results indicate that 18-HEPE is potentially a key metabolite of n-3 PUFAs mediating their repressive effect on CXCR4 expression in B16-F0 melanoma cells.

18-HEPE inhibited melanoma-initiated metastasis

Next, to determine whether 18-HEPE was the specific n-3 PUFA metabolite mediating the inhibition of melanoma-initiated metastasis in vivo, we injected two groups of WT mice via the tail vein with B16-F0 cells previously treated with either 18-HEPE (Pre-18-HEPE group) or with ethanol (control group; Figure 5A, left panel). Prior to injection, the cell viability was not different between two groups. (Supplementary Figure S2B–D, available at *Carcinogenesis* Online). We found that the number of lung tumor colonies in the Pre-18-HEPE group was significantly lower than in the control group (Figure 5A, right panel). Next, to mimic clinical application by maintaining circulating levels of 18-HEPE, 18-HEPE was administered on a daily basis either i.v. or i.p. to mice in which melanoma metastasis had been previously initiated (43) (Figure 5B, left panel). In agreement with our previous results, we found that the numbers of lung tumor colonies in the 18-HEPE intervention groups were significantly lower than in the control group (Figure 5B, right panel). Taken together, our results demonstrate that the suppressive impact of n-3 PUFAs on melanoma metastasis is due to the anti-inflammatory effects of 18-HEPE (Figure 6).

Discussion

Our study provides substantial evidence that pulmonary metastasis of melanoma B16-F0 cells is significantly reduced in Fat-1 transgenic mice, which have elevated lung tissue levels of

Figure 2. Metastasis-induced inflammation is reduced in Fat-1 transgenic mice. (A) MPO activity was an indicator of inflammation levels, and the rainbow color scale indicated the intensity of MPO activity. The color shifted from blue to red indicated the intensity from low to high. Top: the representative image; bottom: the quantitative results of MPO activity. (B) Serum cytokine levels of IL-17 and TNF- α . (C) mRNA expression of TGF β and CXCR4 in lung from WT and Fat-1 transgenic mice. The values were presented as mean $\pm$ SEM ($n = 6-10$). * $P < 0.05$; ** $P < 0.01$.

Figure 3. An increase of EPA metabolite, 18-HEPE in Fat-1 transgenic mice. Serum lipid mediators in WT and Fat-1 mice were analyzed by liquid chromatography-tandem mass spectrometry. (A) The multiple reaction monitoring profiling showed the levels of bioactive lipid mediators and precursors derived from EPA including 5-HEPE, 8-HEPE, 18-HEPE, 19-HEPE, 17,18-diHEPE and 17,18-EpETE. (B) Formation of 18-HEPE from EPA and the serum levels of EPA (C) and 18-HEPE (D). (E) The ratio of AA:EPA was significantly decreased in Fat-1 mice relative to WT mice. The values were presented as mean $\pm$ SEM ($n = 4$). *** $P < 0.001$.

Figure 4. The differential effect of n-3 PUFA derivatives on metastasis target, CXCR4. The melanoma cells were treated with AA or EPA (A) or 18-HEPE (B) for 48 h at indicated concentrations, and CXCR4 protein expression was measured. Data with different letters were significantly different ($P < 0.05$) and were presented as mean $\pm$ SEM.

Figure 5. 18-HEPE intervention blocks melanoma-initiated metastasis. (A) B16-F0 cells were treated with 400 pg/ml 18-HEPE for 48 h and then injected into tail vein. The experimental scheme was shown, and the protein expression of CXCR4 was measured before injection (left panel). The pulmonary metastatic foci were shown in general observation of whole lungs and scatter plot (right panel). (B) 18-HEPE intervention was given to mice after initiating metastasis and continued administration by i.v. and i.p. injection at 100 pg and 1 μ g of 18-HEPE, respectively, until killed (the experimental scheme, left panel). The pulmonary metastatic foci were shown in general observation of whole lungs and scatter plot (right panel). The values were presented as mean $\pm$ SEM ($n = 6-7$). * $P < 0.05$.

Figure 6. The inhibitory role of 18-HEPE on melanoma-initiated metastasis. The diagram illustrates a proposed mechanism by which elevated tissue n-3 PUFA status resulting in an increase of 18-HEPE level downregulates CXCR4, decreasing inflammation and metastasis.

n-3 PUFAs and a balanced n-6:n-3 ratio. This was found to be associated with decreased CXCR4 expression in B16-F0 cells and CXCR4-relevant cytokines and increased levels of the EPA metabolite, 18-HEPE. These results validate the previous observation using mice model fed with diet containing 5% fish oil (27).

In the past decade, epidemiological and experimental studies have shown the differential role of n-3 and n-6 PUFAs in cancer and have specifically emphasized the importance of n-6:n-3 ratio to human health. COX-2, an enzyme that plays a critical role in the conversion of AA to prostaglandins, particularly PGE₂, is now considered a cancer biomarker for prognosis and targeted treatment. On the contrary, n-3 PUFAs, including EPA and DHA, have been shown to exhibit an inhibitory effect on COX-2 activity and PGE₂ production in colon and breast cancer. Our previous data showed that Fat-1 transgenic mice have reduced levels of PGE₂ in primary melanoma, resulting in the delay of tumor growth (30). However, there is little data to distinguish the differential effects of n-6 and n-3 PUFAs on melanoma metastasis.

In this study, we found that n-6 and n-3 PUFAs differentially regulate CXCR4 expression in melanoma cells, suggesting that a lower n-6:n-3 ratio may lead to reduced CXCR4 expression in melanoma. However, a previous study of breast cancer cell lines showed that n-3 PUFAs, though decreased cell surface expression of CXCR4, had no significant effect on overall CXCR4 expression (34). This suggests that n-3 PUFAs may differentially regulate CXCR4 expression depending on the cancer cell type. Previous studies have shown that several lipid mediators of n-3 PUFAs play an inhibitory role in tumor progression (44,45). 18-HEPE and 17-HDHA are hydroxylated metabolites derived from EPA and DHA, respectively; these lipid molecules suppress LPS-induced TNF- α expression of macrophages (41). Interestingly, our results showed that 18-HEPE directly repressed CXCR4 expression of B16-F0 cells. Therefore, 18-HEPE may contribute to inhibiting tumor metastasis, possibly through decreased TNF- α levels. Since a balanced ratio of n-6:n-3 significantly suppresses

tumorigenesis and CXCR4 is now used as a molecular target in cancer therapy, the effect of multiple metabolites on CXCR4 regulation and tumor metastasis requires further investigation.

Additionally, our data demonstrated higher lung TGF β expression in WT mice relative to Fat-1 mice. Although TGF β is a bipotential factor able to promote and suppress tumor growth depending on the stage of the tumor (46), it has been associated with remodeling of extracellular matrix, stimulation of metastasis and induction of CXCR4-dependent cell migration (47). Besides lowering inflammation, n-3 PUFAs play a potential role in coordinating a tumor microenvironment that is refractory to metastasis, including modulating the molecular levels of TGF β and CXCR4. Further supporting data are needed to interpret the mechanism behind how n-3 PUFAs regulate melanoma metastasis via CXCR4.

Over two decades ago, Reich et al. (48) demonstrated that EPA inhibits invasion and metastasis of malignant melanoma cells in lungs *in vitro*. In addition, n-3 PUFAs supplementation was found to attenuate and even to overcome postoperative Natural killer cell suppression, to increase resistance to experimental and spontaneous metastasis and to enhance recurrence-free survival following excision of the metastasizing primary tumors (15). Nevertheless, the efficacy and mechanism of n-3 PUFAs in preventing melanoma metastasis remain inconclusive. Xia et al. (49) found that fish oil enriched with n-3 PUFAs increased both growth and metastatic ability of B16 melanoma cells by suppressing the cytolytic function of both T cells and macrophages. Since it is known that diet is a confounding factor in n-3 PUFAs-related studies, this study might lead to confusion due to poorly controlled diets and the relatively high content of n-3 PUFAs—about 9% compared with 5% in most of studies. To avoid dietary variation, we used the Fat-1 transgenic mouse, which is capable of producing n-3 from n-6 PUFAs endogenously, and this model is known to possess a unique lipidomic profile that is rich in n-3 PUFAs and their relevant metabolites (50). 18-HEPE has been shown to inhibit macrophage-mediated pro-inflammatory activation of cardiac fibroblasts and prevent cardiac pathology (43). This together with our findings suggest that 18-HEPE may play a key role in regulating inflammation and the tumor microenvironment and is potentially a promising anticancer agent. Further investigation of the role played by 18-HEPE may generate new strategies for prevention of melanoma metastasis.

There are a few limitations in our study. Firstly, only metastatic colonies of the lung surface were counted because tumor colonies/cells inside of the lungs could not be readily visualized. Secondly, we were unable to isolate lung tumor cells for further analysis due to the small tumor size of metastatic colonies. Nevertheless, the results present strong validation of n-3 PUFAs' preventative effects on melanoma lung metastasis. To our knowledge, this is the first study to investigate the effect of n-3 PUFAs and their metabolites, 18-HEPE on CXCR4 expression and lung metastasis of melanoma cells.

In summary, our study utilized the Fat-1 transgenic mouse model combined with lipidomic analysis to provide innovative insights into the preventive role of n-3 PUFAs in melanoma metastasis. Our results indicate that increased tissue levels of n-3 PUFAs can decrease lung metastasis of melanoma by increasing the production of 18-HEPE, which can inhibit CXCR4 expression and inflammatory response level. Our findings not only provide new evidence for the anticancer effect of n-3 PUFAs but also has identified a new mechanism for the preventive effect of n-3 PUFAs on melanoma metastasis.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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