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Eicosapentaenoic acid and docosahexaenoic acid inhibit macrophage-induced gastric cancer cell migration by attenuating the expression of matrix metalloproteinase 10

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Abstract

Uptake of docosahexaenoic acid (DHA)/eicosapentaenoic acid (EPA) improves the treatment of cancer and reduces tumor-associated macrophage count. However, the mechanism of this relationship is still unclear.

In this study, macrophages enhanced gastric cancer cell migration ability and induced the differentially expressed matrix metalloproteinase genes (*MMP1*, *MMP3* and *MMP10*) of N87 as identified by polymerase chain reaction array. Furthermore, DHA and EPA inhibited macrophage-enhanced cancer cell migration and attenuated MMP10 at both the RNA and protein level. The suppression of MMP10 expression was further verified by zymography and antibody blocking experiments. Additionally, DHA and EPA attenuated expression of macrophage-activated extracellular-signal-regulated kinase (ERK) and signal transducers and activators of transcription 3 (STAT3) in cancer cells. Attenuation was verified by demonstrating blockade with specific inhibitors and thereby increased MMP10 expression.

Accordingly, we hypothesized that macrophage enhances cancer cell migration through ERK and STAT3 phosphorylation and subsequent increased MMP10 expression and that DHA and EPA could attenuate these signals. These findings not only explain the beneficial effects of DHA/EPA, but also point to ERK/STAT3/MMP10 as the potential targets for gastric cancer treatment.

1. Introduction

Gastric cancer (GC) is the fourth most common cancer worldwide, and almost two thirds of affected individuals will die of their disease [1]. In the majority of tumors, the tumor cell and the macrophage appear to have a symbiotic relationship, with the tumor cell attracting macrophages and prolonging their survival and with the tumor-associated macrophages (TAMs) producing a myriad of factors to promote tumor growth and metastasis [2]. Clinical studies have shown a positive correlation between abundant TAMs and poor prognosis and identified "degree of macrophage infiltration into the cancer cell nest" as a significant predictor of survival in GC patients [3].

It has been suggested that increasing one's intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two main n-3 polyunsaturated fatty acids (n-3 PUFAs), may reduce the risk of chronic diseases [4]. DHA affects cell membrane fluidity and permeability and modifies the activities of cell membrane receptors and enzymes [5,6]. *In vitro* and animal studies indicate that PUFAs suppress colon carcinogenesis by inducing apoptosis [7]. High-dose DHA has been shown to induce apoptosis in GC cells [8] and reported to reduce migration of Treg cells [9]. It thus appears that supplementation with n-3 PUFAs might be a promising therapy for conditions characterized by inappropriate proinflammatory activity [10].

However, the relation of TAMs and GC cells, and the possible effects of DHA and EPA are still unclear. Herein, we present the first evidence in support of the hypothesis that macrophages enhance GC cell migration, and that DHA and EPA inhibit this activity. Moreover, we show that this inhibitory activity might be, in part, mediated by down-regulation of the extracellular-signal-regulated kinase (ERK) and signal transducers and activators of transcription 3 (STAT3) pathways which, in turn, attenuates matrix metalloproteinase 10 (MMP10) expression.

2. Methods and materials

2.1. Cell culture

GC cell lines AGS, N87, MKN45 and TSGH and the monocyte cell line THP-1 were grown in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO_2–95% air, according to the supplier's (American Type Culture Collection, Manassas, VA, USA) recommendations. Adherent cells were detached from the culture dishes with trypsin-EDTA.

THP-1 cells were seeded into culture dishes and induced to differentiate into macrophages by incubation with 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma) for 24 h. The macrophages were washed three times with RPMI medium containing 10% FBS, incubated in this medium for another 24 h to eliminate the effect of TPA and incubated in serum-free media for another 24 h. The harvested and pooled culture supernatants were used as macrophage conditioned medium (CM) as described before [11].

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2.2. In vitro migration assay

Cell migration assays were performed using modified Boyden chambers with filter inserts (pore size 8 μm) in 24-well dishes. Tumor cells were placed in the upper chambers, and the CM from macrophages with/without treatments was added to the lower chambers. Treatments included DHA (Sigma), EPA (Sigma) and neutralizing anti-human-MMP10 antibody (R & D Systems, Minneapolis, MN, USA). After overnight exposure, the cells in the upper chamber were fixed with methanol, stained with 1% crystal violet and counted under a microscope.

2.3. Cell viability assay

The cytotoxicity of DHA and EPA was determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). GC cells were co-cultured with various concentrations of DHA and EPA for 24 h, and their viability was determined using a standard MTT protocol.

2.4. RNA extraction, polymerase chain reaction (PCR) array and reverse transcriptase (RT)-PCR

After culture with macrophage CM for 6 h, GC cells were collected, and their total RNA was extracted using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Extracellular matrix and adhesion molecule PCR arrays (SA Biosciences, Frederick, MD, USA) were performed according to a previously described protocol [12]. Total RNA (3 μg) was reverse transcribed using oligo(dT) primers and M-MLV reverse transcriptase (Invitrogen). cDNAs were amplified by PCR using primers for MMP1, MMP3, MMP10 [13], ITGAM, ITGB1 and GAPDH. The inhibitors U1026, LY294002 (Sigma) and STAT3 Inhibitor V (Stattic; Merck, Darmstadt, Germany) were used to determine the interaction between the MAPK and STAT3 pathways and MMP10 RNA expression.

2.5. Western blot analysis

Cells were washed with phosphate-buffered saline, scraped into radioimmunoprecipitation assay buffer and centrifuged. The supernatants (cell lysates) were subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were stained using primary antibodies specific for MMP10 (R&D Systems), β-actin, ERK, p-ERK, Akt, p-Akt (Santa Cruz Laboratories, Santa Cruz, CA, USA) and p-STAT3 (Cell Signaling, Beverly, MA, USA) and then a secondary antibody (Santa Cruz). Specific signals were visualized using a chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, England).

2.6. Zymography

Gelatinase activity was measured in conditioned medium by zymography. This procedure has been shown to estimate both proenzyme and activated MMP enzyme activity. Equal amounts of conditioned medium were subjected to electrophoresis on 10% zymography gels containing 0.1% gelatin (Novex, San Diego, CA, USA). Gels were washed with renaturing buffer (Novex) for 30 min, incubated in developing buffer (Novex) overnight at 37°C and stained with Coomassie blue.

2.7. Statistical analysis

Results are presented as mean±standard error of the mean (S.E.M.). All statistical analysis was conducted using the statistical package SPSS 13.0. The significance of differences was evaluated using the Student's *t* test and one-way analysis of variance. Values of *P* were considered to be statistically significant if $P<.05$, $^{**}P<.01$.

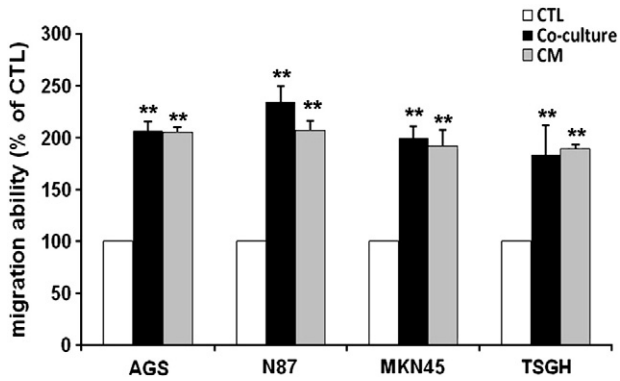
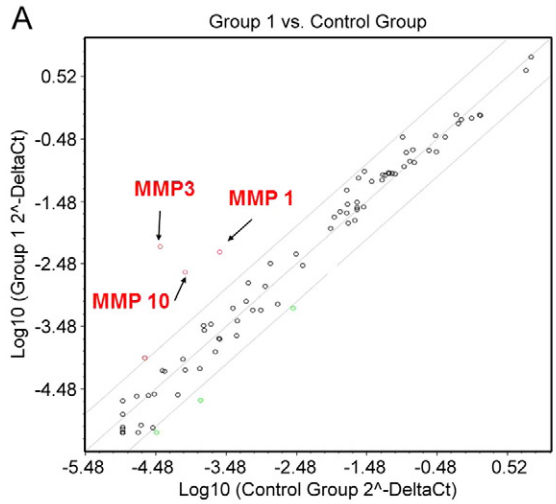


Fig. 1. The migration ability of GC cells treated by macrophage CM. Four GC cell lines (AGS, MKN45, N87 and TSG1) were seeded into Boyden chambers and co-cultured with or without macrophage cells or macrophage CM for 24 h. The ability of each cell line to migrate was measured by a Boyden chamber-based migration assay after 24 h of incubation. All data are expressed as the arithmetic mean±S.E.M. $^{**}P<.01$.



B

gene	fold change	gene	fold change	gene	fold change
MMP3	160.4522	RPL13A	1.3068	PPC	-1.458
MMP10	27.5886	THBS1	1.271	MMP2	-1.5094
MMP1	18.7134	ADAMTS1	1.2277	LAMC1	-1.5411
ITGAM	4.426	TIMP2	1.2193	PPC	-1.6518
ITGB1	3.3311	SPP1	1.2025	HAS1	-1.6865
ITGA2	3.3081	CTNNA1	1.1942	COL11A1	-1.6982
LAMB3	3.1296	TGFBI	1.1859	VCAN	-1.6982
CD44	2.9814	COL15A1	1.1455	CTNND2	-1.6982
MMP7	2.4217	ECM1	1.0989	FN1	-1.6982
ITGA7	2.4049	CTNND1	1.0913	ITGA4	-1.6982
MMP13	2.1825	ITGA3	1.0762	ITGA8	-1.6982
COL6A2	1.8869	ITGAV	1.0614	KAL1	-1.6982
TNC	1.7975	CTGF	1.0182	MMP16	-1.6982
ITGB2	1.7851	ITGAL	-1.0168	MMP8	-1.6982
ICAM1	1.7728	ACTB	-1.06	NCAM1	-1.6982
MMP14	1.7124	TIMP1	-1.0673	SELP	-1.6982
LAMB1	1.6771	COL6A1	-1.105	THBS2	-1.6982
SPG7	1.654	VTN	-1.1127	TIMP3	-1.6982
ITGB4	1.6088	B2M	-1.136	VCAM1	-1.6982
CLEC3B	1.6088	CNTN1	-1.1439	HGDC	-1.6982
ITGA1	1.5867	CTNNB1	-1.1519	PPC	-1.71
RTC	1.5867	SELL	-1.1761	COL7A1	-1.795
LAMA3	1.4804	ITGA6	-1.2092	ADAMTS13	-1.9643
CDH1	1.46	COL16A1	-1.2176	COL5A1	-2.0056
RTC	1.4499	MMP9	-1.2176	MMP12	-2.3198
ITGB5	1.4399	PECAM1	-1.2959	COL12A1	-2.3359
ITGB3	1.43	MMP15	-1.314	COL8A1	-2.521
ADAMTS8	1.4103	THBS3	-1.314	SPARC	-2.8363
RTC	1.3435	LAMA2	-1.3232	COL14A1	-3.7685
HPRT1	1.3342	ITGA5	-1.351	MMP11	-4.4816
COL1A1	1.325	SELE	-1.3986	SGCE	-5.1124
COL4A2	1.3159	GAPDH	-1.4479	LAMA1	-6.5614

Fig. 2. PCR array analysis of the effect of macrophage CM treatment. N87 cells were cultured in the presence or absence of macrophage CM for 6 h. Extracellular matrix and adhesion molecule mRNAs were analyzed by PCR microarray (SA Biosciences). Fold change greater than 3.33 is indicated as significant.

3. Results

3.1. Macrophage induces gastric cancer cell migration

Analysis of the migration of four gastric cancer cell lines (AGS, N87, MKN45 and TSGH) found an almost twofold increase in activity after co-culture with macrophage or treatment with CM for 24 h (Fig. 1).

3.2. PCR array identified differentially expressed genes of N87 cells after co-culture with macrophage

PCR array was used to identify genes up-regulated by exposure to macrophage CM. Three genes (*MMP1*, *MMP 3* and *MMP 10*) were significantly up-regulated (27-, 160-, and 18-fold, respectively) after treatment (Fig. 2A and B). The up-regulation of these genes was later verified at the RNA and protein levels (Fig. 4).

3.3. DHA and EPA inhibited migration of N87 cells

DHA and EPA were shown to induce apoptosis of human gastric cancer cells [8,14]. To verify the effect of low-dose DHA and EPA on GC cell (N87) migration and viability, cells were treated with various doses of DHA and EPA (0–50 μM) for 24 h. MTT assay (Fig. 3A) found that 80% of cells remain viable in the presence of 5 μM DHA and EPA.

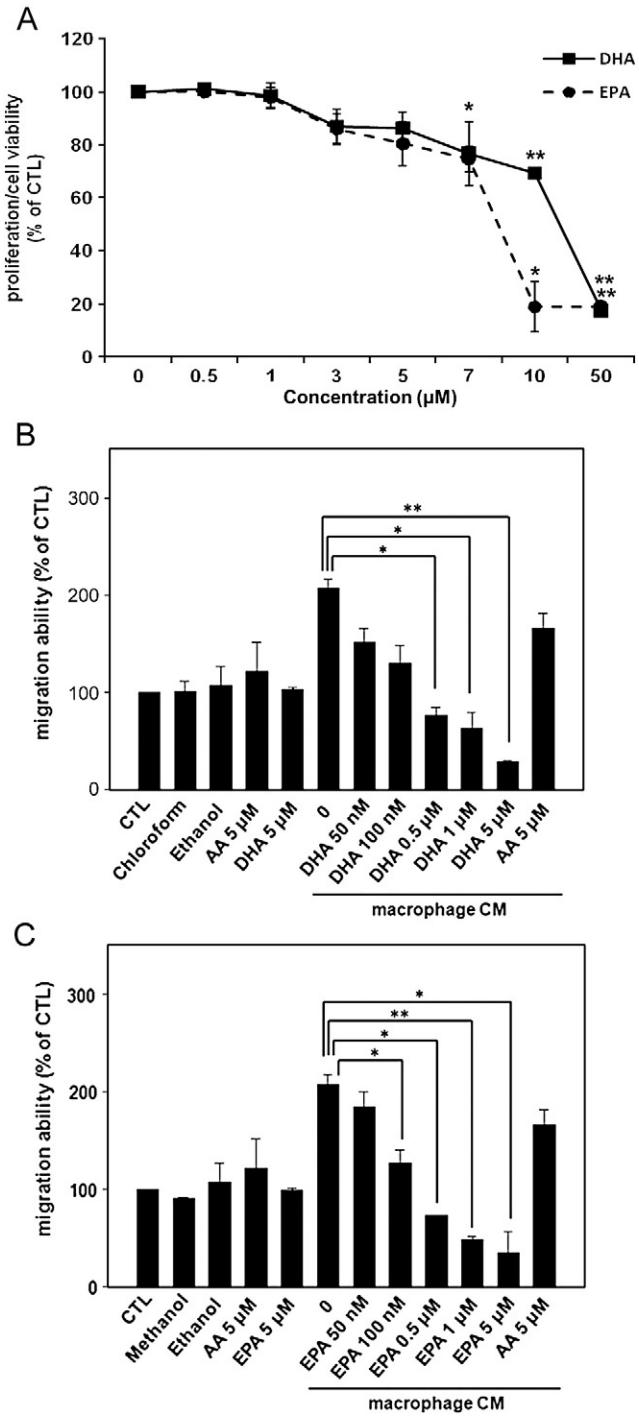


Fig. 3. Effect of DHA and EPA on gastric cancer cells treated with macrophage CM. (A) The viability of N87 cells treated with macrophage CM and DHA or EPA at various doses (0, 0.5, 1, 3, 5, 7, 10 and 50 μM) for 24 h was determined by MTT assay. The ability of cells exposed to macrophage CM and various doses (0, 0.05, 0.1, 1 and 5 μM) of DHA (B) or EPA (C) to migrate was measured *in vitro* after incubation in Boyden chambers for 24 h. All data are expressed as the arithmetic mean±S.E.M. *P<.05; **P<.01.

CM-induced N87 cell migration was dose-dependently suppressed by DHA (0–5 μM) from 207% to 29% (Fig. 3B) and EPA (0–5 μM) from 207% to 35% (Fig. 3C) but not the n6-PUFA arachidonic acid (5 μM), indicating that DHA and EPA significantly inhibit macrophage-activated cell migration. We have conducted the migration study with AGS cell line also and found that DHA and EPA inhibited migration abilities of not only N87 but also AGS. The two cell lines have the same trend of behavior in the experimental model of this study (data not shown).

3.4. DHA and EPA inhibit macrophage-activated cell migration by down-regulation of MMP10

To determine whether DHA and EPA inhibited macrophage-activated gastric cancer cell migration by down-regulating the expression of genes identified as significantly up-regulated by PCR microarray (i.e., *MMP1*, *MMP3* and *MMP10*), the levels of the corresponding RNAs in CM-treated cells were measured before and after exposure to 5 μM DHA and EPA for 24 h. Only MMP10 was down-regulated (Fig. 4A). Similarly, MMP10 protein expression (assayed by Western blot using anti-MMP10 antibodies) showed 25% and 41% down-regulation in the presence of 5 μM DHA and EPA, respectively (Fig. 4B). Furthermore, zymography showed that MMP10 activity in culture medium collected after 24-h treatment with 5 μM DHA and EPA was significantly decreased compared with CM only (Fig. 4C), indicating that both DHA and EPA inhibited MMP10 gene expression and decreased secretion of pro-MMP10 and active MMP10. To further confirm the relationship between migration and MMP10, the ability of neutralizing antibody to block MMP10 activity was tested. Cell migration was significantly decreased from 248% to 78% by pretreatment with 10 μg/ml anti-MMP10 (Fig. 5), suggesting the involvement of MMP10 in macrophage-activated GC cell migration.

3.5. DHA- and EPA-mediated decrease in MMP10 expression might lead to the down-regulation of the ERK and STAT3 pathways

It was reported that AKT and MAPK are involved in many signal transduction pathways [15], macrophage-conditioned medium triggers the ERK cascade [16], and expression of MMP10 is regulated by the ERK [17] and STAT3 pathways [18]. In this study, macrophage CM induced ERK, AKT, JNK and STAT3, and DHA and EPA attenuated phosphorylation of ERK, AKT and STAT3 (Fig. 6A). To confirm the interaction between the MAPK/STAT pathway and MMP10, cells were pretreated with the ERK inhibitor (U0126), AKT inhibitor (LY294002) or STAT3 inhibitor (Stattic). MMP10 RNA expression was significantly inhibited by U0126 and Stattic (Fig. 6B), suggesting that MMP10 was regulated by ERK and STAT3 but not AKT.

4. Discussion

TAMs play an important role in malignancy. Most reports show a significant correlation between the density of TAMs and poor prognosis [2]. In the present study, macrophage and macrophage CM caused a significant increase in GC cell migration. It has been reported that recruitment of TAMs is due to cancer-cell-mediated up-regulation of host stromal cell production of colony-stimulating factor-1 [19]. TAMs stimulate tumor growth, produce angiogenic factors [20] and facilitate vascular invasion of tumor cells [21,22]. It has also been reported that the invasiveness of tumors co-cultured with macrophages is enhanced by tumor necrosis factor-α and MMPs released from macrophages [23–26].

Lipids are not only a source of energy, but some lipids modulate metabolic and inflammatory responses. N-3 PUFAs are preferentially incorporated into cell membrane phospholipids, influence secondary messenger synthesis and modulate the expression of certain adhesion

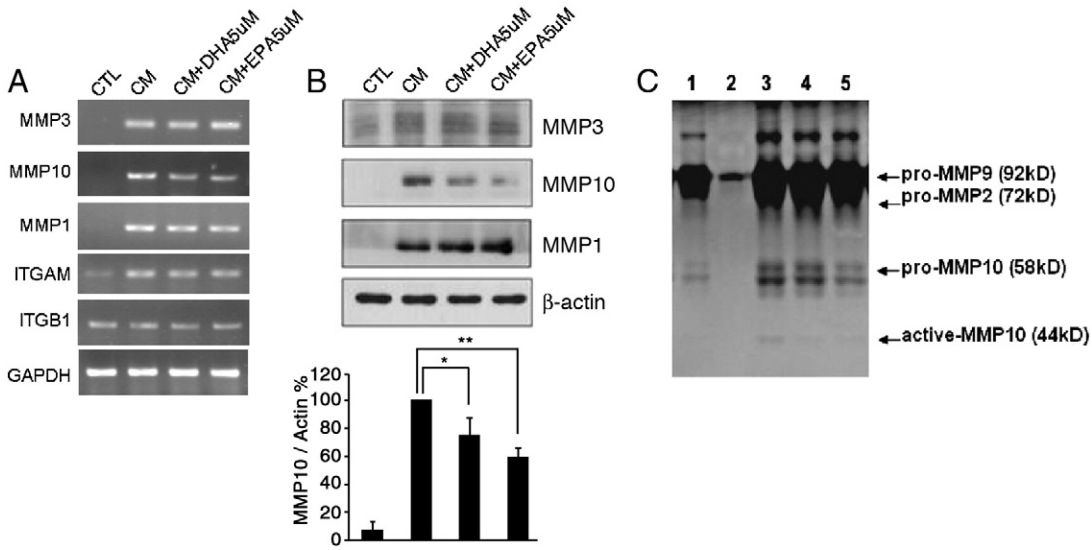


Fig. 4. Effect of DHA and EPA on MMP mRNA and protein expression. N87 cells in the presence or absence of macrophage CM were treated with or without 5 μM of DHA or EPA. (A) mRNA level was measured by RT-PCR in cells collected after 6 h of treatment. (B) Protein level was measured by Western blot in cells collected after 24 h of treatment. Western blot data are expressed as the arithmetic mean±S.E.M. *P<.05; **P<.01. (C) Activity of MMPs released into the conditioned medium by 24-h exposure to fish oil was measured by gelatin zymography; fish oil treatment resulted in decreased activity of both pro and active MMP10 (lane 1, macrophage conditioned medium control and untreated N87 cells; lane 2, control; lane 3, macrophage conditioned medium; lane 4, macrophage conditioned medium with DHA 5 μM; lane 5, macrophage conditioned medium with EPA 5 μM).

molecules at the surface of endothelial cells, monocytes and lymphocytes [27–30].

In our study, two major n-3 PUFAs, DHA and EPA, significantly inhibited TAM-activated migration of GC cells. It is compatible with previous results where n-3 PUFAs has been shown to trigger anticancer activity in cell models [31,32]. In either case, the mechanisms are of obvious interest clinically even if some randomized controlled trials found little evidence showing that higher intake of omega 3 fatty acids affects the incidence of cancer [33]. It is likely that suppression of tumor cell growth by n-3 PUFAs is due to a combination (rather than one) of these mechanisms [32,34–37]. It has been proposed that n-3 PUFAs included in the diet are incorporated into cell membranes of normal and tumor tissues where they serve as substrate for COX-2 and inhibit the expression of the inflammation-producing and growth-promoting prostaglandin E2 [37]. As cancer growth depends on development of new blood vessels to supply nutrients and to remove wastes, inhibition of angiogenesis may inhibit or limit tumor growth [38–41].

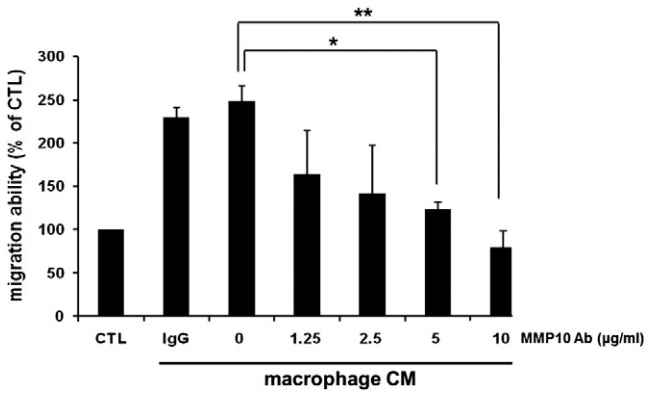


Fig. 5. Effect of MMP10 neutralizing antibody on migration of GC cells. N87 cells exposed to macrophage CM for 24 h were treated with different concentrations of MMP10 neutralizing antibody (0, 1.25, 2.5, 5 and 10 μg/ml) for 30 min. Isotype IgG was used as a blocking control. All data are expressed as the arithmetic mean±S.E.M. *P<.05; **P<.01.

MMPs induce extracellular matrix breakdown to permit normal tissue remodeling and are involved in tissue destruction in arthritis, cancer invasion and metastasis [42,43]. Microarray analysis showed that co-culture with macrophages up-regulates the expression of MMPs in gastric cancer cells [44]. In this study, PCR microarray revealed significant up-regulation of MMP1, MMP3 and MMP10 in gastric cancer cells treated with macrophage CM. The up-regulation of MMPs was confirmed by RT-PCR and Western blot. Interestingly, macrophage CM-induced up-regulation of MMP10 (but not MMP1 and MMP3) was significantly attenuated in the presence of DHA and EPA. MMP10 is expressed in epithelial cells [45] but not in fibroblasts [46,47]. Overexpression of MMP10 has been reported in cancers of the lung, head and neck [48], esophagus [49,50], brain [51] and liver [52]. It was demonstrated that MMP10 is a good marker for detection of GC and is a prognostic factor for GC [53]. MMP10 activates proMMP7 and proMMP9 [54], which are thought to be particularly important for the malignant behavior of GC cells [55,56]. Thus, MMP10-specific inhibitors may have a role in the treatment of various types of cancer, including GC. MMP10 expression was modulated through transcriptional regulation in most cases [57–59]. To date, there is no known posttranscriptional modification of MMP10. However, expressions of other MMPs were known to be suppressed through miRNA-dependent translational suppression. For example, translational activity of MMP2 was suppressed by miR29b binding, and MMP9 was suppressed through miR491-5p [60,61]. MMP10 might also be regulated in a similar way. The involvement of MMPs in cancer invasion and metastasis has been reported [42]. In our study, GC migration was significantly inhibited by MMP10 neutralizing antibody. MMP10 is important in modulating basement membrane degradation and thereby may facilitate tumor progression [62–65]. Therefore, MMP10 could be a target for GC therapy.

Several studies have indicated that the signaling proteins including MAPK members, PI3K and AKT are involved in the expression of MMPs and including metastasis [66–69]. In order to evaluate whether DHA or EPA mediates and/or inhibits the MAPK members and AKT, the effect of DHA or EPA on the phosphorylated status of ERK 1/2, JNK 1/2 and AKT in gastric cancers was investigated. In this study, macrophage CM induced ERK, AKT, JNK and STAT3, but DHA/EPA only attenuated phosphorylation of ERK, AKT and STAT3.

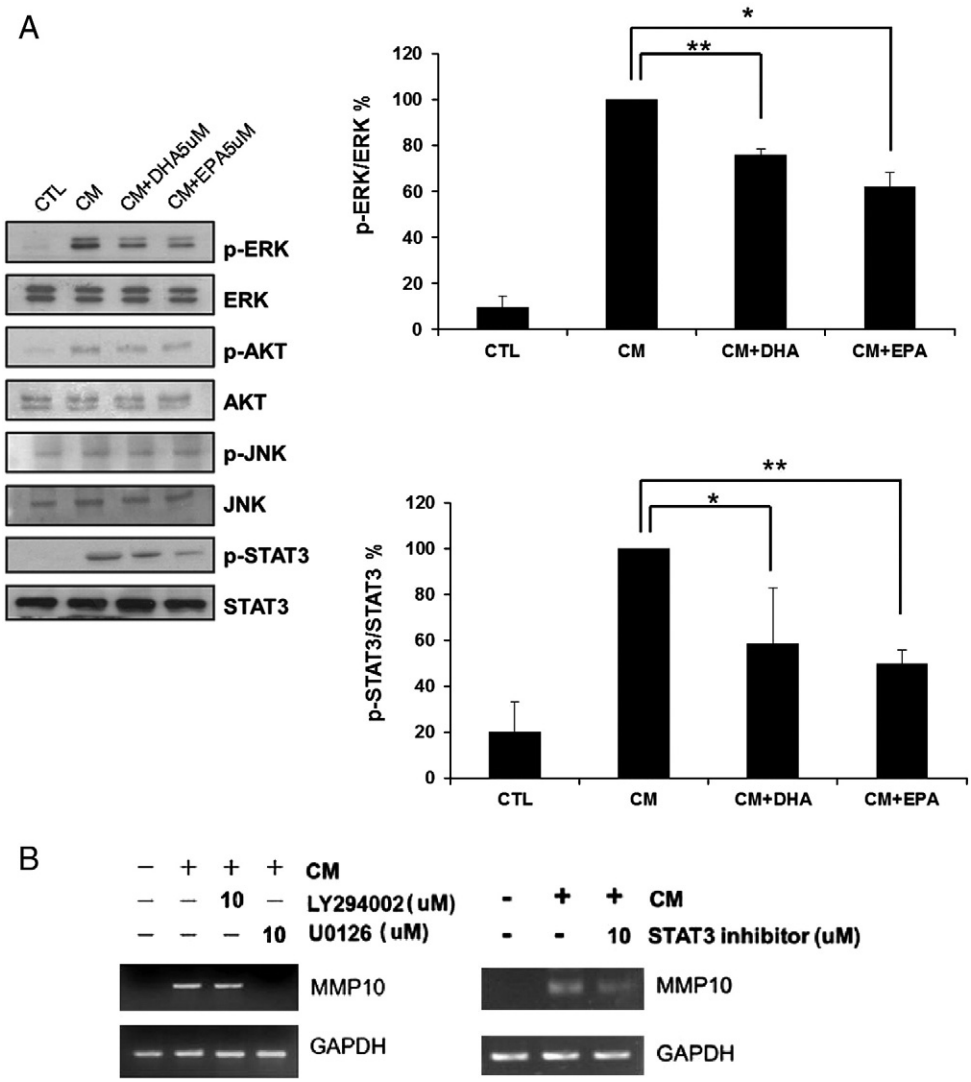


Fig. 6. Effect of DHA and EPA on the MAPK pathway. N87 cells exposed to macrophage CM for 30 min were treated with 5 μM DHA or EPA for 1 h. (A) Rates of ERK, AKT, JNK and STAT3 phosphorylation were determined by Western blot. (B) The effect of inhibitors LY294002, U0126 and STAT3 inhibitor (10 μM for 1 h) on the expression of the MMP10 gene in N87 cells exposed to macrophage CM for 6 h was determined by RT-PCR. All data are expressed as the arithmetic mean±S.E.M. *P<.05; **P<.01.

We also confirm the involvement of ERK, AKT and STAT in DHA/EPA-inhibited MMP-10 expression by specific inhibitors (Fig. 6B). Our results indicate that DHA and EPA regulate MMP-10 through suppressed phosphorylation of ERK and STAT3 but not AKT and JNK.

It is consistent with the recent report that macrophage-conditioned medium triggers the ERK pathway [16], which regulates MMP10 expression [17]. Moreover, it has been shown that STAT3 activation plays a role not only in MMP1 and MMP10 induction by epidermal growth factor in T24 bladder cancer cells [70], but also in interleukin-6 mediated MMP10 expression in human lung adenocarcinoma cancer cell lines [18]. In our investigation, phosphorylation of ERK and STAT3 was induced by macrophage CM and attenuated by both DHA and EPA, suggesting that the attenuation of TAMs-induced MMP10 expression by n-3 PUFAs might be mediated through MAPK and STAT3 regulation.

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