

Docosahexaenoic acid reduces in vitro invasion of renal cell carcinoma by elevated levels of tissue inhibitor of metalloproteinase-1

Anthony J. McCabe^{a,*}, Julie M.W. Wallace^b, William S. Gilmore^b,
Hugh McGlynn^a, Sean J. Strain^b

^aCancer and Aging Research Group, University of Ulster, Coleraine, UK

^bNorthern Ireland Centre for Diet and Health (NICHE), University of Ulster, Coleraine, UK

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Abstract

We demonstrate in this study that the n-3 polyunsaturated fatty acids derived from fish oil, namely, eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), can increase levels of tissue inhibitors of metalloproteinase-1 (TIMP-1) in the renal cell carcinoma cell line caki-1 by 26% and 17.42% respectively. The result of this elevation in TIMP-1 levels is a reduction of 48.48% in caki-1 invasion through the basement membrane component matrigel when cells are treated with DHA. By inhibition of 2-series prostaglandin production, a similar increase in TIMP-1 was observed in caki-1 cells. We conclude that the polyunsaturated fatty acid DHA, a component of fish oil, is capable of significantly reducing the invasive profile of renal cell carcinoma, and that this reduction is regulated by levels of 2-series prostaglandin production.

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1. Introduction

Health benefits of fish consumption have long been linked to the intake of the n-3 polyunsaturated fatty acid (PUFA) components of fish oil. Numerous population studies have shown that incidences of western diseases such as coronary heart disease and cancer were much higher in western populations compared to Alaskan and Greenland Eskimo populations, and this difference has been attributed to high fish content in the diet of these peoples [1,2]. Fish, and particularly oily fish, are high in n-3 PUFA, of which eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) are active constituents.

There is an accumulating body of evidence that suggests that dietary intervention with polyunsaturated fatty acids can influence both the growth and metastatic potential of numerous cancers in vivo. Investigators have reported reduced tumor load and number and also tumor metastases

for cancers of the mammary gland [3], colon [4], and lung [5] after consumption of diets rich in n-3 PUFA, as well as an enhancement of tumor load and metastases after diets supplemented with n-6 polyunsaturated fatty acids.

Metastasis is the most life-threatening aspect of cancer and is responsible for the high mortality rates among cancer patients. Tumor metastasis involves a complex series of biochemical interactions during which cancerous cells escape from the primary tumor, migrate, and form distant metastases elsewhere at target organs. During metastasis, cancer cells adhere and interact extensively with components of the extracellular matrix (ECM), via integrin adhesion molecules, expressed at the surface of cells. At this point of contact, matrix metalloproteinases (MMPs) enable the cancer cell to degrade the various components of the ECM, thus completing the initial escape from the site of the primary tumor.

The MMPs are zinc metallo-endopeptidases. MMPs were initially described as regulators of cell invasion through their ability to degrade extracellular matrix components. MMPs are inhibited by endogenous inhibitors known as the tissue inhibitors of metalloproteinases

* Corresponding author. Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06510, USA.

E-mail address: anthony.mccabe@yale.edu (A.J. McCabe).

(TIMP). Although in recent years many nonmatrix substrates have been found to be targets of MMP activity and alternative, nonmatrix regulating functions of MMP and TIMPs have been described including roles in proliferation and angiogenesis [6,7], arguably their most important role is in regulating cell invasion through matrix degradation. Numerous studies have demonstrated correlation between expression and production of MMPs and the TIMPs and tumor growth, metastasis, and angiogenesis [8–11]. Gelatinase A or matrix metalloproteinase-2 (MMP-2) is one member of the MMP family that can degrade the extracellular matrix components fibronectin, collagen, and elastins [12].

Polyunsaturated fatty acids have been implicated in the control of MMP-2 expression. In 1993, Rose et al. demonstrated the differential effects of n-6 and n-3 PUFA on the degradation of basement membrane components in vitro by a human breast cancer cell line [13]. In this study, and in further in vivo animal studies, it was demonstrated that n-3 PUFA have an inhibitory effect on the production of MMP-9 and subsequent metastasis in mice [14,15]. Conversely, in a similar study, n-6 PUFAs were demonstrated to have a stimulatory effect on MMP-9 production in vitro [16], and this effect was attributed to eicosanoid production from n-6 PUFA metabolism.

Worldwide mortality from kidney cancer has been expected to exceed 100,000 persons early in the new millennium [17], and cancer of the kidney accounts for 2% of all cancer in Northern Ireland [18]. Renal cell carcinoma accounts for 80–85% of all renal malignancies. Nearly one half of all patients diagnosed with renal cell carcinoma die within 5 years of diagnosis, and the 5-year survival rate of those patients with metastatic disease is only 5–10% [19]. Traditional therapies such as radiotherapy and chemotherapy have not proved useful in the treatment of renal cell carcinoma and surgery remains the only alternative treatment. In this study, we investigate the effect of the n-6 PUFA arachidonic acid and its metabolites, as well as the n-3 PUFA eicosapentanoic acid and docosahexaenoic acid, on MMP-2 and its inhibitor TIMP-1 and on the invasive profile of a renal carcinoma cell line *caki-1*. (The results suggest that manipulation of dietary n-3 PUFA may offer new possibilities for the prevention and treatment of renal cell carcinoma.)

2. Methods and materials

2.1. Cell culture

The human renal cell carcinoma cell line, *caki-1*, was obtained from the European collection of Animal Cell Culture (ECACC, Salisbury, UK). *Caki-1* cells were routinely maintained in McCoy's 5A media (Life Technologies, Glasgow, UK) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, UK). Cells were grown at 37°C at 5% CO₂.

2.2. Fatty acid preparation

The n-3 PUFAs EPA and DHA and the n-6 PUFA AA were obtained in the 99% pure free acid form from Sigma Chemical (UK). Working solutions of PUFAs were made by dissolving in 100% ethanol. Polyunsaturated fatty acids were added to *caki-1* cells in McCoy's 5A media containing 10% fetal bovine serum. The inhibitors of the cyclooxygenase pathway, palmityl trifluoromethyl ketone (PTK), indomethacin (INDO), ketoralac (Ket) and picotamide (PICO), were all purchased from Sigma (UK) and resuspended to stock concentrations in 100% ethanol.

2.3. MMP and TIMP measurements

Caki-1 cells were grown in the PUFA concentrations indicated for a period of 24 hours and supernatants were removed. For MMP-2 measurement, supernatants were diluted 1:50 and for TIMP-1 measurements, supernatants were diluted 1:5. Supernatants were diluted in 0.3 mol/L phosphate chloride assay buffer. MMP-2 and TIMP-1 were measured using the Biotrack ELISA system (Amersham, UK).

2.4. Matrigel invasion assay

Invasion chambers were prepared as follows 200 µg of matrigel basement membrane protein (Becton Dickinson, UK) was diluted in 200 µL of serum free McCoy's 5A media. The matrigel was pipetted onto a 4.2-cm² membrane insert (pore size 8 µmol/L) and allowed to dry. The final concentration of matrigel on each insert was 50 µg/cm². Before use, the chambers were rehydrated by adding 500 µL of serum free media for 2 hours. Once rehydrated, the chambers were emptied and 5 × 10⁴ *caki-1* cells, previously grown in fatty acids for a 24-hour period, were added into each chamber with 1.5 mL of serum free media. The inserts were placed in six-well plates and 1.5 mL of 10% fetal calf serum added to the wells as a chemoattractant. The chambers were incubated for 24 hours at 37°C at 5% CO₂. After 48 hours, the cells remaining on the upper surface of the inserts were removed with cotton swabs. The cells, which had invaded to the underside of the insert, were fixed in ethanol and stained with hematoxylin stain (Sigma, UK). The numbers of invading cells were counted under a light microscope.

2.5. Prostaglandin E₂ measurements

Intracellular levels of prostaglandin E₂ produced in *Caki-1* were measured using an enzyme immunoassay Biotrack (Amersham, UK). *Caki-1* cells (1 × 10⁵) were grown in 96-well plates. After indicated treatments, cells were lysed in 100 µL of lysis buffer. Quantities of 50 µL of the lysates were measured for PGE₂. Total PGE₂ concentrations per well (1 × 10⁵ cells) were determined by extrapolation from a standard curve.

3. Results

3.1. TIMP levels are effected by PUFA

The levels of TIMP-1 and MMP-2 produced and secreted by caki-1 cells in response to fatty acid supplementation was assessed by ELISA. EPA was added at 30 $\mu\text{mol/L}$ concentration, and DHA and AA were used at concentrations of 10 $\mu\text{mol/L}$. These concentrations were found to have no effects on caki-1 proliferation, as measured by the MTT proliferation assay (data not shown). All experiments involving PUFA supplementation were at these concentrations for 24 hours.

Supplementing caki-1 cell media with EPA, DHA, and AA had no effect on MMP production under the above-mentioned experimental conditions (Fig. 1b); however, these PUFAs produced differing effects on the TIMP-1. TIMP-1 production was significantly increased by caki-1 cells supplemented with EPA, which increased total TIMP-1 levels by 26% and DHA, which increased total TIMP-1 levels by 17.42% (Fig. 1a).

3.2. n-3 PUFA decrease cell invasion through matrigel

Caki-1 cells grown in 10 μM MDHA resulted in a significant decrease in the invasion of these cells through

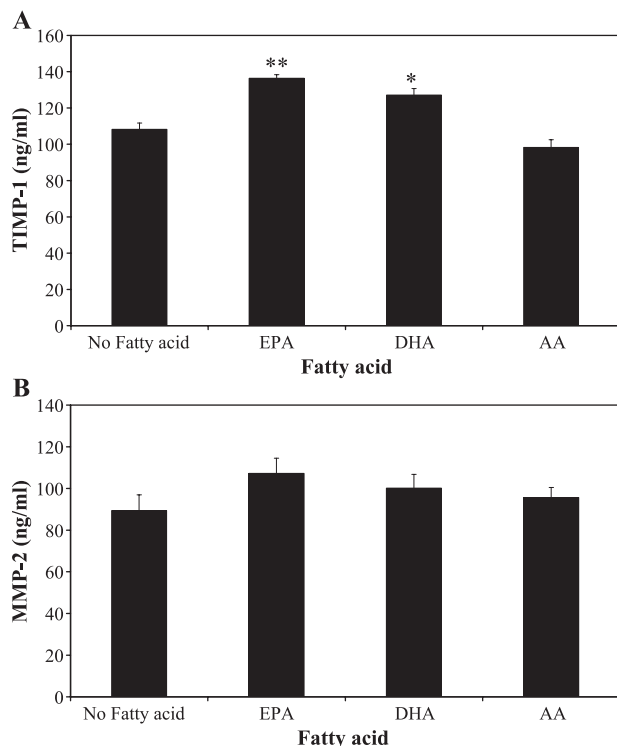


Fig. 1. The effects of polyunsaturated fatty acids on the production of tissue inhibitor of metalloproteinase-1 (TIMP-1) (A) and matrix metalloproteinase-2 (MMP-2) (B). Caki-1 cells were grown for 24 hours in the fatty acids indicated. The supernatants were removed and measured for MMP-2 and TIMP-1. The results represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.005$ with the Student t test. Results are representative two independent experiments performed in triplicate.

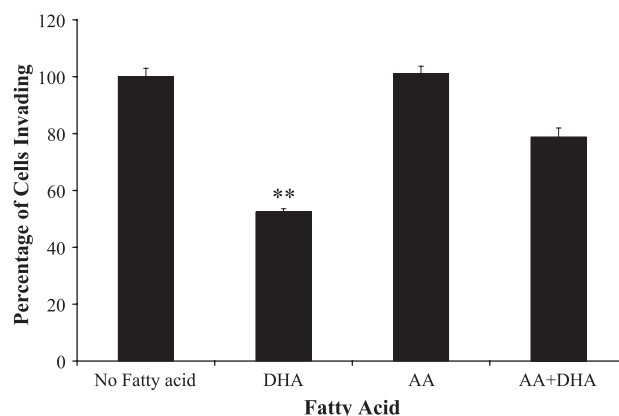


Fig. 2. Caki-1 cells were grown for 24 hours in the fatty acids combinations illustrated. The cells were subsequently removed and plated on inserts coated with matrigel basement membrane compound. Numbers of stained caki-1 cells invading through matrigel after 24 hours. Experiments were performed twice in triplicate and number of cell counted represents the mean of 10 random fields of view under $20\times$ magnification. * $P < 0.005$ with the Student t test.

the basement membrane component substrate matrigel. The addition of 10 $\mu\text{mol/L}$ AA alone had no effect on caki-1 cell invasion. However, when added with DHA, AA offset the reduction in invasion induced by DHA. This suggests that AA is involved in regulating the anti-invasive activity of DHA on caki-1 cells, probably through modulation of TIMP levels (Fig. 1). The addition of EPA to caki-1 cells also reduced caki-1 cell invasion through matrigel by up to 29%; however, these decreases were not significantly different from controls (data not shown).

3.3. TIMP-1 levels are regulated by prostaglandin production

Fig. 1 demonstrates that DHA affect the levels of TIMP-1 produced in caki-1 cells and also that this fatty acid results in a reduction in invasion through matrigel, acting possibly through the inhibition of MMPs. The question arises of how these fatty acids regulate TIMP-1 levels. In an attempt to address this question, we incubated caki-1 cells with inhibitors of 2-series prostaglandin production, which are products of arachidonic acid metabolism and which have previously been shown to affect MMP levels. We postulated that the same may be true for TIMP expression. Both n-3 and n-6 polyunsaturated fatty acids are primary substrates for cyclooxygenase, which catalyzes the initial step in the formation of 2-series prostanoids. Although n-3 fatty acids are the preferred substrate over n-6, the sheer abundance of AA, an n-6 fatty acid, in cell membranes means that AA is a readily available substrate for cyclooxygenase and hence more often metabolized (Fig. 2).

Fig. 3 shows that inhibition of AA metabolism at several points. Inhibition at each step of arachidonic acid metabo-

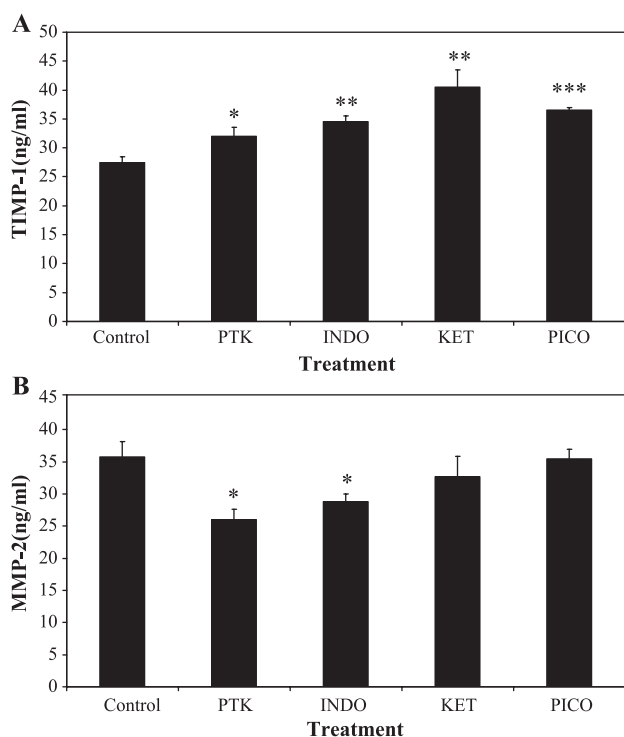


Fig. 3. Caki-1 cells were grown in the presence of inhibitors of the cyclooxygenase pathway. 5 $\mu\text{mol/L}$ palmityl trifluoromethyl ketone (PTK), 10 $\mu\text{mol/L}$ indomethacin (INDO), 1 $\mu\text{mol/L}$ ketoralac (KET), and 5 $\mu\text{mol/L}$ picotnamide (PICO). Cells were incubated in each inhibitor for 24 hours prior to assaying for MMP-2 and TIMP-1. Experiments were performed twice in triplicate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ with the Student t test.

lism produced a significant elevation of total TIMP-1 levels. PTK reduces free AA release from membranes and hence available substrate for metabolism by inhibiting phospholipase A_2 activity, and this inhibition is enough to produce significant increases in TIMP-1. Indomethacin, which inhibits cyclooxygenase activity and KET and PICO inhibit prostaglandin H_2 synthase and thromboxane A_2 synthase activity respectively. By inhibiting these enzymes, the production of prostaglandin E_2 and thromboxane A_2 was reduced or inhibited completely, resulting in dramatic increases in TIMP-1 (Fig. 3a). Conversely, addition of indomethacin and PTK reduce levels of MMP-2 production in caki-1 cells (Fig. 3b).

We propose that DHA reduces invasion of caki-1 cells primarily through elevated TIMP-1 production, it does this by reducing free AA available for cyclooxygenase activity, resulting in reduced prostaglandin E_2 and thromboxane A_2 synthesis, which we have demonstrated to affect total TIMP-1 production. As shown in Fig. 4, DHA is capable of reducing prostaglandin E_2 to levels seen when cells are treated with indomethacin, a specific cyclooxygenase inhibitor; this indicates that this fatty acid derived from fish oils can effectively reduce the levels of such immunomodulatory compounds as efficiently as cyclooxygenase reducing drugs.

4. Discussion

We report in this article that n-3 and n-6 PUFA can affect levels of MMP and TIMP-1 and that these effects are regulated by prostaglandin production. The n-3 fatty acids EPA and DHA, which are abundant fatty acids in fish oils, are capable of increasing levels of TIMP-1 in the renal cell carcinoma cell line caki-1. Neither EPA or DHA, however, had any affect on total MMP-2 levels, as measured by ELISA. The resultant altered MMP:inhibitor ratio produces a reduced invasive phenotype in the epithelial cell line caki-1, as demonstrated by a reduction in the number of invading cells through matrigel basement membrane.

Levels of both MMP and TIMPs have previously been shown to be influenced by the addition of exogenously added prostaglandins [20–22] and that cyclooxygenase activity can be manipulated by fatty acids to induce these changes [24]. We believe that the changes we report in TIMP levels are regulated by endogenous 2-series prostaglandins, the levels of which are controlled by the metabolism of PUFA. The 2-series prostaglandins are products of n-6 PUFA metabolism by a pathway-initiated release of arachidonic acid from membrane phospholipid pools by phospholipase A_2 and subsequent oxidative metabolism by cyclooxygenase. DHA has been shown to be a potent inhibitor of 2-series prostaglandin production [29]. The exact mechanism whereby DHA exerts its effects on prostaglandin production are far from clear, but a number of mechanisms have been suggested. Retroconversion of DHA to EPA and competition with AA for cyclooxygenase [30], decreased expression of cyclooxygenase [31], and inhibitory activity for phospholipase A_2 [32] have all been suggested.

We inhibited n-6 PUFA metabolism by the addition of several pathway inhibitors. We showed that inhibition at each step of the AA metabolism pathway leading to

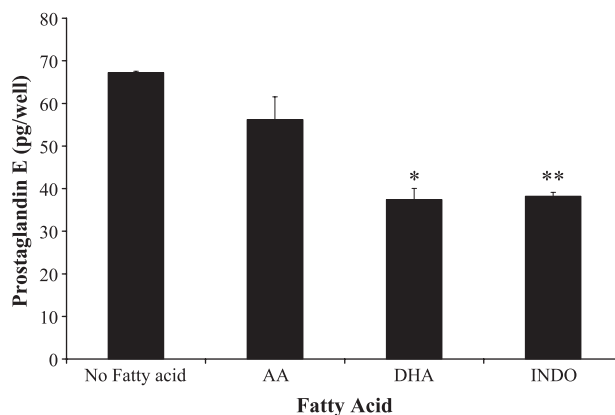


Fig. 4. Regulatory effect of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) on the production on 2-series prostaglandins. Caki-1 cells were grown in the fatty acids indicated and also the cyclooxygenase inhibitor indomethacin for 24 hours. Prostaglandin 2 levels were measured by ELISA. Experiments were again performed twice and in duplicate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ with the Student t test.

prostaglandin production produces significant increases in TIMP-1 levels in the renal cell carcinoma caki-1 cells, and we postulate that these effects are due to the reduction in 2-series prostaglandin levels. We hypothesize that n-3 PUFA modulate TIMP-1 production in this manner by successfully competing with AA for cyclooxygenase activity or inhibiting AA reduction, and by the addition of DHA to the cells, we would see a reduction in arachidonic acid metabolites. We tested our hypothesis by demonstrating that prostaglandin E₂ production is reduced upon addition of exogenous DHA and that this inhibition in turn elevates TIMP-1 levels. We have also demonstrated that addition of DHA to the cells is equally as effective at decreasing intracellular prostaglandin E₂ levels when compared to the cyclooxygenase inhibitor, indomethacin.

The MMPs, which are pivotal to the growth and progression of cancer, are obvious targets for development of anticancer therapies [24]. Changes that alter the tightly regulated balance between MMPs and their inhibitors, TIMPs, could have diverse effects on the prognosis of many pathological conditions such as cancer.

Recently, dietary intervention with n-3 PUFA has been shown to reduce levels of these proteinases in several different cancers in rat and mouse models [3,21,25]. Furthermore, in a prospective study in men, modulation of n-3/n-6 ratio in the diet has been shown to reduce the risk of progression of prostate cancer [26]. Our data are limited to MMP-2 and TIMP-1. Although other studies have shown effects of n-3 polyunsaturated fatty acids on MMP-9, our cell line caki-1 produces almost undetectable levels of MMP-9. However, with as many as 30 members of the MMP family and four tissue inhibitors of MMPs (TIMP-1 through TIMP-4), further work is necessary to study the scope of these observed effects of DHA and eicosapentanoic acid, on MMPs and their inhibitors in other renal cell lines and also a range of cancer cell lines of epithelial lineage. However, our data do add further evidence that dietary intervention with fish oils that are high in n-3 PUFA may be considered as an optional adjuvant therapy alongside traditional therapeutic regimens in treating renal cell carcinoma growth and progression, a disease the progression of which has been associated with elevated MMP levels [25], particularly given that the concentrations used in our study are within physiological levels of DHA [33]. The action of the n-3 PUFA in vitro suggest possible anti-metastatic properties. It has been suggested, however, that intake of n-3 PUFA alone may not be sufficient to obtain all of the possible health benefits of dietary intervention without additional reduction in total intake of n-6 PUFA [26–28]. In our laboratory we have shown that treatment of caki-1 cells with AA alone reduces levels of TIMP-2 (data not shown), so clearly there are effects of arachidonic acid on TIMP and/or MMP production. Levels of AA should therefore be given consideration in any alteration of dietary intake of polyunsaturated fatty acids, the suggestion being that a balance between n-3 and n-6 PUFA in the diet is

perhaps a more critical parameter rather than total intake of one PUFA class over another.

Acknowledgments

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