n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE$_2$ induced ERK-1 and -2 and HIF-1$\alpha$ induction pathway

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Abbreviations: COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic; ERK, extracellular signal-regulated kinase; HIF1-$\alpha$, hypoxia-inducible factor 1-$\alpha$; LI %, Labeling Index; PUFAs, polyunsaturated fatty acids; RIA, radioimmunoassay; VEGF, vascular endothelial growth factor.

Introduction
A series of studies have demonstrated that n-3 polyunsaturated fatty acids (PUFAs) have many beneficial effects in neoplastic pathology, ranging from the decrease of tumor growth (1), inhibition of metastasis (2), reduction of cachexia (3) and attenuation of various side effects of different chemotherapeutic agents (4). Their anti-angiogenic potential has also recently been put forward on the basis of the observations that n-3 PUFA enriched diets decreased the number of microvessels developing in tumors derived from mouse (5) or human (6) mammary cell lines transplanted in mice. Vascular endothelial growth factor (VEGF) is considered one of the most closely associated factors in the induction of the neovascularization of human colon cancer. In these cancers VEGF is self-produced by the neoplastic cells whose VEGF levels are elevated and correlated with a poor clinical outcome (7). However, increased levels of VEGF protein and mRNA are observed not only in advanced stages of cancer but also in pre-malignant stages of tumor development (8). We have found previously that the proliferation of colonic mucosa pre-neoplastic lesions, such as sporadic adenomas in adenomatous polyposis, can be inhibited by supplementation of patients with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (9,10). Also, human colon cancer cells growing in nude mice have been shown to be highly sensitive to the growth inhibitory action of n-3 PUFAs (11,12). On the basis of these observations we designed this study in order to investigate in human colon cancer cells the effect of EPA or DHA treatment on tumor growth and VEGF production in vitro and in vivo. This was done by analyzing EPA and DHA effects on VEGF expression and secretion in human HT-29 colon cancer cells growing in culture. These cancer cells express constitutively VEGF, and we have employed an experimental model in which the production of this factor was augmented by serum starvation (13). EPA and DHA modulation of the levels of cyclooxygenase-2 (COX-2) and PGE$_2$, recently implicated as inducers of angiogenesis in colon cancer, was also investigated. The activation of extracellular signal-regulated kinase (Erk-1) and Erk-2, and the expression of the hypoxia-inducible factor 1-$\alpha$ (HIF-1$\alpha$) protein, shown to be important mediators for up-regulation of VEGF expression, were also assessed. Finally, the effects of a dietary treatment with these fatty acids on VEGF induction was studied on HT-29 cells growing in Balb/c nude mice. To this purpose tumor growth, microvessel formation, expression of VEGF and COX-2 and PGE$_2$ levels were evaluated.

Materials and methods

Cell culture
The human colon adenocarcinoma cell line, HT-29, was obtained from the American Type Culture Collection (ATCC, USA) and cultured in minimal essential medium supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM l-glutamine, vitamins and non-essential amino acids in a humidified atmosphere at 37$^\circ$C under 5% CO$_2$. The cells were maintained in log phase by seeding twice a week at a density of 5.5 x 10$^3$ cells/cm$^2$ and the experiments were performed 1 day after trypsinization. In order to increase the constitutive VEGF expression of HT-29 cells, all the experiments were carried out in serum-deprived condition, by excluding FCS from standard culture.
medium. EPA and DHA (Sigma, St Louis, MO) were added at the start of the experiments from an ethanol stock solution and control cells were treated with the same amount of vehicle alone. In order to prevent oxidation, fatty acids were stocked under nitrogen and stored at 80°C.

**Animals and treatments**

Forty-five male athymic Balb/c nude mice, aged 6 weeks, were housed in cages within a pathogen-free isolation facility and fed a non-purified commercial diet (Altromin–Rieper, Bolzano, Italy). The animals were randomly divided into three treatment groups (15 mice/group). Groups 1 and 2 received daily, by gavage, a dose (1 g/kg body wt) of EPA or DHA ethyl ester, respectively. Fatty acid ethyl esters were kindly provided by Hoffmann-La Roche (Basel, Switzerland). The control group (group 3) received water instead of n-3 PUFAs ethyl esters by gavage in the same amount to avoid possible effects due to gavage procedure. The amount of water given to animals by gavage was ~20 μL/mouse/day.

After 7 days of treatment the animals were inoculated sub-axillary with 10⁶ viable HT-29 cells (human colon adenocarcinoma line cell), and the treatment lasted until the end of the experiment. The 7-day pre-treatment was performed because we found previously (14) that this is the time required to obtain the maximal incorporation of EPA and DHA in mouse tissues. The tumors became palpable 6–8 days after transplantation and were measured with a caliper every 3 days (the tumor volume was calculated as 0.5 × length × width²). Mice from each group were killed 28 days after HT-29 cells transplantation by cervical dislocation and the tumors were excised, weighed and sectioned. Tumor sections were fixed in 10% buffered formalin for immunohistochemical examination. Paraffin-embedded tumor sections were deparaffinized and the slides were washed in PBS and incubated overnight at 4°C with anti-VEGF monoclonal (Santa Cruz Laboratories, Santa Cruz, CA; 1:80 dilution), Ki67 (Clone MIB-1, Dako Cytomation, Denmark; 1:100 dilution) and COX-2 polyclonal (Cayman Chemical, Ann Arbor, MI; 1:20 dilution) antibodies. The slides were then subjected to 1 h room temperature with peroxidase-conjugated secondary antibodies washed, incubated with DAB and counterstained with hematoxylin. Negative controls were performed with the same procedure but without the primary antibody. Five microscopic fields from different sections were observed and the percentage of positively stained cells was determined from cell counts made at high-power magnification (400×). To evaluate tumor microvessel formation tumor sections were stained for CD31 using the anti-CD31 monoclonal antibody (Novocasta, Newcastle, UK; 1:50 dilution).

**Detection of apoptosis**

The percentage of apoptotic cells was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (18). Paraffin-embedded tumor sections were deparaffinized and incubated for 5 min with hybridization buffer (Boehringer, Mannheim, Germany). The sections were then incubated with 2.5 U of TdT and 100 pmol biotin-dUTP in hybridization buffer for 1 h at 37°C. Thereafter the sections were incubated with the streptavidin-biotin-peroxidase complex (Sigma, St Louis, MO) for 30 min at room temperature. The sites of peroxidase binding were detected with DAB and the sections were counterstained with hematoxylin. Negative controls in the absence of TdT were also performed to verify the aspecific reading due to peroxidase. Five microscopic fields from three different sections were observed and the percentage of TUNEL-positive apoptotic cells (Labeling Index, LI %) was counted at 400× magnification.

**Results**

**HT-29 cells in vitro**

HT-29 cells showed a scarce constitutive VEGF production, however, they were strongly induced to enhance their VEGF production by serum starvation, confirming the finding of Jung et al. (13). Forty-eight hours was the serum starvation period required to obtain the maximal amount of VEGF production by HT-29 (Figure 1A). The treatment with increasing concentration of EPA (10–30 μM, final concentration) inhibited VEGF expression induced by serum starvation in a concentration-dependent manner (Figure 1B) with an estimated IC₅₀ of 13.1 μM. Also, DHA (10–30 μM) showed similar effects (Figure 1C), even though its efficacy was lower than that of EPA, as shown by its higher IC₅₀ concentration (19.6 μM). Moreover, whereas 30 μM EPA was sufficient to inhibit VEGF expression almost totally (by 90.3 ± 6.2%), 30 μM DHA reduced VEGF expression by only 60.2 ± 4.3%. The inhibition of VEGF expression was also accompanied by a decreased secretion of this factor, as assessed by ELISA of VEGF levels in the culture medium of HT-29 cells.
Also in this case EPA showed higher effectiveness than DHA.

In these conditions HT-29 cell growth, evaluated by cell counting (Figure 2A and B), was significantly inhibited by both EPA and DHA in a concentration-dependent manner. EPA showed higher cell growth inhibitory efficacy than DHA (30 μM, 73.3 ± 3.7% and 56.6 ± 4.9% inhibition, respectively).

As recently it has been shown that colon cancer cells over-express COX-2 (19) which, in turn, induces the production of various angiogenic factors, including VEGF (20), and that n-3 PUFAs may act as regulators of the expression of the inducible enzyme COX-2 in several experimental models (21–23), we investigated the effect of a 24-h EPA and DHA treatment on COX-2 expression in serum-deprived HT-29 cells. We chose this length of treatment as preliminary experiments showed that the maximal COX-2 expression was after 24 h of serum starvation (data not shown). Both EPA and DHA (Figure 3A and B) inhibited COX-2 expression, even though the EPA effect was much more pronounced than that of DHA (30 μM EPA: 36.8 ± 3.5% inhibition; DHA 27.8 ± 1.9% inhibition).

As one of the products of COX-2 metabolism, PGE2 has been implicated recently as an inducer of VEGF and angiogenesis in human colon cancer (24), we investigated the alterations in PGE2 levels in supernatants obtained from HT-29 cells grown in serum-free conditions for 24 h (Figure 3C). In this case we used the concentration of EPA and DHA (30 μM), which revealed the higher efficiency in the previous experiments. Both the fatty acids reduced the production of PGE2, but EPA demonstrated higher efficacy than DHA (reduction: EPA 28.2 ± 5.1%; DHA 37.1 ± 6.0%).

To verify the hypothesis that EPA and DHA down-regulate VEGF expression specifically inhibiting COX-2-induced PGE2 production, we measured VEGF expression in HT-29 cells simultaneously treated with n-3 PUFA and exposed to exogenous PGE2 (0.1 μM) (Figure 4). We found that in this condition high levels of VEGF expression were restored, suggesting that the n-3 PUFA down-regulate VEGF expression by inhibiting PGE2 production.
We next evaluated extracellular signal-regulated kinase (ERK) phosphorylation, in HT-29 cells after serum starvation and EPA and DHA treatments. This was done because it was reported in HT-29 cells that ERK activation is a key step in the up-regulation of VEGF induced by serum starvation (13). Moreover, it was reported recently that PGE2 exposure induces ERK activation in colon cancer cells, and that the pharmacological inhibition of ERK phosphorylation prevents the induction of VEGF mRNA in response to PGE2 stimulation (24). We found a concentration-dependent inhibition of ERK-1 and -2 phosphorylation induced by 24 h serum starvation by both EPA and DHA (Figure 5A and B). Also in this case, EPA was much more efficient than DHA (30 µM, 58.0 ± 5.1 and 30.2 ± 2.8% inhibition, respectively). As shown in the same figure total (phosphorylated plus unphosphorylated) Erk-1 and -2 expression was not affected by n-3 PUFA treatments, indicating that EPA and DHA specifically reduced the phosphorylation process.

As it has been shown recently that PGE2-induced VEGF expression in colon cancer cells is mediated by the transcriptional activator hypoxia-inducible factor-1 (HIF-1) through the PGE2-stimulated expression of inducible HIF-1α protein (24), we investigated whether HIF-1α protein expression in HT-29 cells is stimulated by serum starvation and whether EPA and DHA are able to modify the expression of this protein. We found a time-dependent increase of HIF-1α expression in serum-free conditions, with a maximal induction at 24 h (data not shown). Both EPA and DHA inhibited HIF-1α expression in a dose-dependent manner (Figure 5C and D), being EPA (IC50: 26.5 µM) much more efficient than DHA (IC50: 42.5 µM).

Furthermore, PGE2 (0.1 µM) reduced the effects of EPA and DHA on HIF-1α and phosphorylated Erk-1 and -2 expression in serum-starved HT-29 cells. In these cells, 30 µM EPA or DHA completely returned HIF-1α and phosphorylated Erk-1 and -2 levels to the control values, further demonstrating that...
n-3 PUFAs are able to reduce the COX-2/PGE2/pErk-1,-2/HIF-1α transducing pathway of angiogenesis through the inhibition of PGE2 production.

HT-29 cells transplanted in vivo

The doses of EPA and DHA supplemented (1.0 mg/g body wt) did not affect the amount of food consumed daily by the animals. Also, weight gain, before tumor implantation, was not significantly different (ANOVA) among the groups of animals. Even though during tumor growth total body weight (actual body weight + tumor weight) was affected by tumor size, no significant differences in weight gain were found at necropsy, when subtraction of tumor weight from body weight was performed for each animal (data not shown). EPA and DHA decreased significantly the growth of HT-29 cells implanted subcutaneously in Balb/c nude mice, as shown in the macroscopic photograph (Figure 6A), reducing tumor volume, measured at the 28th day after implantation, by 58.2 ± 6.3 and 51.1 ± 5.6%, respectively (Figure 6B). Similarly, tumor weight, measured at excision, was decreased by 42.8 ± 4.6 and 41.0 ± 4.4%, in animals supplemented with EPA and DHA (Figure 6A).

The percentage of proliferating cells in tumors (LI %, Table I), determined immunohistochemically using antibodies to Ki67, was significantly decreased by both EPA and DHA treatments (decrease: 56.5 ± 4.6 and 46.5 ± 3.8%, respectively).

Moreover, an increase in the percentage of apoptotic cells (LI %, Table I), analyzed by TUNEL assay, was noticed in tumors from both EPA- and DHA-treated mice (increase: 162.4 ± 12.8 and 148.4 ± 12.7%, respectively).

In order to evaluate whether n-3 PUFA dietary treatment modified microvessels formation in HT-29 derived tumors we assessed tumor microvessel density by immunohistochemistry using antibodies against CD31 (Figure 7). The figure shows...
that EPA treatment markedly reduced the number of microvessels per field (48.2 ± 6.1%, decrease). A similar reduction was observed also when the mice were supplemented with DHA (data not shown).

Since the in vitro experiments showed that EPA and DHA were able to inhibit VEGF and COX-2 expression, we also evaluated immunohistochemically the expression of these proteins also in tumors of control and treated mice (Figure 8A and B). We found that both n-3 PUFA s inhibited with similar efficacy VEGF (by 28.2 ± 2.3 and 33.6 ± 2.8%, respectively) and COX-2 expression (by 32.5 ± 3.8 and 37.0 ± 3.5%, respectively). Also, PGE$_2$ levels (Figure 8C), measured in tumor homogenates, were markedly reduced by treatments with EPA and DHA (42.8 ± 3.7 and 41.0 ± 4.5% decrease, respectively), consistently with the results obtained in vitro.

**Discussion**

In the present work we demonstrate that EPA and DHA are able to inhibit cell growth and VEGF expression in HT-29 human colon cancer cells cultured in vitro as well as in vivo when implanted in nude mice. The identification of antiangiogenic factors appears crucial for colorectal cancer therapy, as VEGF expression in these tumors has been related with scarce prognosis (7). n-3 PUFA s appear to be optimal candidates, as they are able to reduce the incidence of colon cancers among populations consuming a large quantity of n-3 PUFA s (25,26). Moreover, they inhibit carcinogen-induced colon tumorigenesis in rats (27) and reduce the growth of transplantable colon carcinoma implanted in mice (28,29).

We demonstrate that they are able to inhibit cell proliferation and induce apoptosis in HT-29 cells growing in vivo. The proapoptotic effect of n-3 PUFA s observed by us in HT-29 cells growing in vivo confirm the findings of Clarke and colleagues obtained in the same colon cancer cells cultured in vitro (30). Furthermore, they are able to inhibit microvessel formation in the tumors derived from HT-29 cells transplanted in nude mice.

Both EPA and DHA reduce VEGF expression and secretion in HT-29 cells cultured in vitro, but they do not show similar effectiveness, EPA being much more efficient than DHA. On the contrary, the effects exhibited by both the fatty acids on VEGF expression in vivo is quite similar. It is possible that the lower in vitro efficiency of DHA as compared with EPA may be compensated in vivo by the different metabolism of DHA, known to be the most represented n-3 PUFA in mammalian cell membranes (31,32).

We sought to investigate the transduction pathways involved in PUFA-induced inhibition of VEGF expression in colon cancer. VEGF is a potent angiogenic factor with a recognized major role in the promotion of new vessel formation in colon cancer (33). Colon cancer cells produce constitutively VEGF, and many factors implicated in the induction of this atypical VEGF production by tumor cells have been identified, including autocrine activity of tyrosine kinase growth factor receptors, such as EGFR or IGF-1R (34,35), various genic alterations (involving p-53, ras and src) (35,38) and lower supply of oxygen and growth factors (13,39). Moreover, recently it has been proven that COX-2, one of the enzymes

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**Fig. 8.** Effects of EPA and DHA dietary treatment on VEGF and COX-2 expression and PGE$_2$ levels in tumors obtained from HT-29 cells transplanted in athymic Balb/c mice. Treated mice received a daily dose of EPA or DHA ethyl ester (1 g/kg body wt). (A and B) The expression was evaluated by immunohistochemical analysis, as described in the Materials and methods. (C) RIA was used to analyze tumor homogenates, as described in the Materials and methods. Values are means ± SE of at least six determinations (A and B) and of five different experiments, each performed in triplicate (C). *Significantly different from control (P < 0.05, one-way ANOVA, followed by Fisher’s test).
involved in prostaglandin biosynthesis, can modulate the production of angiogenic factors, including VEGF, by colon cancer cells (20). The expression of this enzyme is normally undetectable in intestinal cells, but appears elevated in up to 85% of colorectal adenocarcinomas (40). We found that EPA- and DHA-induced reduction in VEGF expression may be related to the parallel reduction of COX-2 protein expression in HT-29 cells both in vitro and in vivo conditions. This decreased COX-2 protein expression confirms that observed previously in chemically induced colorectal tumors of rats exposed to a high fish oil diet (27) and in HT-29 and Caco-2 cells treated in vitro with n-3 PUFAs (41,42). Preliminary experiments demonstrated also that COX-2 mRNA expression was significantly reduced in serum-starved HT-29 cells incubated in the presence of EPA and DHA (data not shown), confirming the results obtained by various authors using different models, such as rat mammary glands, rat smooth muscle cells and mouse spleen (43-45).

We found that the COX-2-reduced expression was accompanied also by decreased levels of PGE2 in the supernatants of HT-29 cells cultured in the presence of EPA or DHA and in tumors of PUFAs-treated mice. The data are in keeping with those of Dommels and colleagues who demonstrated recently an EPA-dependent inhibition of arachidonic acid-induced PGE2 production in HT-29 cells (41). PGE2 is one of the COX enzymic products recognized recently as an inducer of VEGF expression in human colon cancer (24). The ability of exogenously added PGE2 to prevent EPA and DHA inhibitory effect on VEGF expression in HT-29 cells suggests that COX-2/PGE2 pathway is involved in the anti-angiogenic action of these fatty acids. Moreover, we found that EPA and DHA are able to markedly inhibit ERK-1 and -2 phosphorylation, which has been identified as an obligatory step for up-regulation of VEGF induced by serum starvation in colon cancer cells (13). The reduced phosphorylation of ERK-1 and -2 in the presence of EPA and DHA substantiates the hypothesis that these fatty acids modulate the COX-2/PGE2 pathways of VEGF expression, as recently it has been reported that ERK activation is a critical step in the signaling pathways leading to the over-expression of VEGF in PGE2-stimulated colon cancer cells (24).

It has been shown that the transcriptional activation of the VEGF gene by PGE2 is mediated by HIF-1, and when this transcription factor is activated, the expression of its inducible subunit HIF-1α protein is increased (24,46). Further confirmation that n-3 PUFA reduction of VEGF expression involves the modulation of COX-2/PGE2 angiogenic pathway is the finding that EPA and DHA reduce the expression of HIF-1α in HT-29 cells cultured in serum-starved conditions. Moreover, the observation that the addition of exogenous PGE2 is able to restore HIF-1α and phosphorylated ERK-1 and -2 expression to control levels in n-3 PUFA-treated cells further supports the modulation of the COX2/PGE2/pERK/HIF-1 angiogenic pathway by these fatty acids. Additional substantiation is also provided by the observation that EPA is always more efficient than DHA not only in reducing the levels of VEGF, but also in decreasing those of COX-2 and HIF-1α, and in inhibiting the phosphorylation of ERK-1 and -2.

Overall, these results indicate that the COX-2/PGE2/ERK/HIF-1 pathway of VEGF expression is likely to be a chief target of n-3 PUFA action. Moreover, they suggest a possible clinical application of these fatty acids as anti-angiogenic compounds in colon cancer therapy.

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References


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