An enhanced apoptosis and a reduced angiogenesis are associated with the inhibition of lung colonisation in animals fed an *n*-3 polyunsaturated fatty acid-rich diet injected with a highly metastatic murine melanoma line

Antonella Mannini, Nadja Kerstin, Lido Calorini, Gabriele Mugnai and Salvatore Ruggieri*

Department of Experimental Pathology and Oncology, University of Florence, Viale G.B. Morgagni 50, 50134 Florence, Italy

Both epidemiological and experimental studies indicate that dietary *n*-3 PUFA inhibit carcinogenesis and tumour growth. Metastatic diffusion has also been found to be affected in animals fed diets containing purified *n*-3 PUFA or fish oil. In the present study, we investigated whether the metastatic diffusion of a highly metastatic variant (F10-SR cells) isolated from the B16 melanoma F10 line was affected by feeding host animals a diet containing 5 % fish oil. In these animals, compared with those fed a diet containing 5 % maize oil, there was a reduced number of metastatic pulmonary colonies. The immunohistochemical analysis of appropriate markers revealed that the antimetastatic effect of dietary *n*-3 PUFA was not related to a reduction of proliferation, but rather to an enhanced apoptotic activity. The reduction of von Willebrand factor immunoreactivity found in pulmonary colonies of F10-SR cells grown in fish oil-fed animals indicates that a decrease of angiogenesis contributes to the antimetastatic effect of dietary *n*-3 PUFA. This conclusion stands in spite of the higher expression of vascular endothelial growth factor observed in pulmonary colonies grown in fish oil-fed animals.

B16 melanoma line: Fish oil diets: Metastases: Apoptosis: Angiogenesis

Although epidemiological data support a strong positive correlation between certain tumours and high-fat diets[1,2], it is still debated how a high consumption of dietary lipids affects carcinogenesis. Animal studies provide evidence that a high content of linoleic acid (18 : 2*n*-6) in the diet influences the development of carcinomas of the breast, colon and prostate, by acting at the promotional stage of carcinogenesis (for a review, see Carroll & Khor[3]). The reproduction of experimental metastasis has also been shown to be favoured by 18 : 2*n*-6-rich diets[4–7]. These experimental data prompted interest in 18 : 2*n*-6 as a possible factor promoting the development of human tumours, in view of the fact that 18 : 2*n*-6 is the most abundant fatty acid of the *n*-6 series in vegetable oils which are the major lipid component in the diets of the Western world. The promoting activity of 18 : 2*n*-6 on carcinogenesis and tumour progression appears to be mediated by the bioactive metabolites of arachidonic acid (AA), the product of elongation and desaturation of 18 : 2*n*-6[8,9].

More recently, epidemiological studies have reported that the risk of breast[10], colon[11] and prostate[12] cancers is lower in populations whose dietary habits are characterised by a high intake of fish oil, which is particularly rich in EPA and DHA, the higher homologues of the α-linolenic acid (18 : 3*n*-3) family[13]. The protective effect of *n*-3 PUFA may also be derived from the observation of an enhancement of breast cancer rate[14] among Japanese women during the past decades associated with a replacement of fish oil with maize oil in their diet. The recognition of an inhibitory activity of *n*-3 PUFA on tumour development was further supported by animal studies showing that feeding diets enriched with *n*-3 PUFA inhibited spontaneous and chemically induced carcinogenesis[15–17], growth of mammary[18–20], colon[21] and liver carcinomas[22] in rats and mice, and growth of human lung carcinoma in nude mice[23].

Metastatic diffusion of melanoma[24], colon[25], mammary[26] and Lewis lung[27] carcinomas was also inhibited when they were transplanted into animals fed *n*-3 PUFA-rich diets. In these studies, however, a few histotypes of malignant cells have been considered, and the effects of *n*-3 PUFA on metastatic diffusion have been examined by using various experimental approaches, for example, by feeding diets containing fish oil[26] or purified EPA and DHA in variable proportions[25], and by subcutaneous administration of fish oil[28]. Moreover, there are also a few studies that demonstrated a promoting activity of dietary *n*-3 PUFA on tumour growth and experimental metastasis[28,29]. Thus, more information should be available before introducing the use of appropriate dietary formulations for the chemoprevention and chemosuppression of human tumours.

To expand our knowledge about the inhibitory effects of dietary *n*-3 PUFA on metastatic diffusion, in the present study we investigated to what extent a diet containing 5 % fish oil, compared with a diet containing 5 % maize oil, affects the reproduction of experimental metastases developed from F10-SR

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cells, a highly metastatic variant isolated from the murine B16 melanoma F10 line. In the present study, we also investigated whether an alteration of the metastatic potential of F10-SR cells induced by our dietary protocol involves some biological activities crucial in tumour progression, such as cellular proliferation[30], apoptosis[31,32] and angiogenesis[31,33]. Cellular proliferation, apoptosis and angiogenesis were examined by immunolocalisation of their respective markers in metastatic pulmonary colonies developed from F10-SR cells transplanted into animals fed diets containing maize oil or fish oil. These markers were: proliferating cell nuclear antigen (PCNA)[34], bax-bcl2[35], vascular endothelial growth factor (VEGF)[36] and endothelial-associated von Willebrand factor (vWF)[37].

Materials and methods

Cells and culture conditions

Cells used in the present study were a highly metastatic variant (F10-SR cells) isolated in our laboratory, from a metastasis reproduced by the murine B16 melanoma F10 line in adrenal gland. F10-SR cells (5×10^5) were seeded in 100 mm tissue culture dishes (Sarstedt), and grown in Dulbecco's modified Eagle's medium containing glucose (4500 mg/l) supplemented with 10 % fetal calf serum (Gibco) at 37°C in a 10 % CO_2 humidified atmosphere. Cell cultures were propagated every 3 d by trypsinisation.

Diets

Diets used in the present study were: (a) a diet composed of 70 % sucrose, 16 % casein, 3 % α-cellulose, 1 % choline chloride, 1 % vitamins, 4 % salt mixture and 5 % maize oil (maize oil diet); (b) an isoenergetic diet in which 5 % maize oil was replaced with 5 % fish oil (fish oil diet). The major fatty acids of the maize oil diet were: 14 : 0, 5·3 %; 16 : 0, 1·6 %; 16 : 1, 16·7 %; 18 : 0, 3·3 %; 18 : 1, 22·1 %; 18 : 2, 50·0 %. The fish oil diet contained: 14 : 0, 21·1 %; 16 : 0, 31·6 %; 16 : 1, 10·5 %; 18 : 0, 5·0 %; 18 : 1, 18·6 %; 18 : 2, 3·0 %; 20 : 5*n*-3, 5·5 %; 22 : 6*n*-3, 4·5 %. The maize oil and fish oil diets, supplied by Laboratorio Dottori Piccioni (Milan, Italy), were stored at 4°C in sealed plastic bags *in vacuo*.

Animals and dietary treatments

Newly weaned female mice (C57Bl/6 strain; Charles River, Frederick, MD, USA) were placed on the maize oil diet for 1 week, and then divided in two groups, each of six animals: one group continued to be fed the maize oil diet, while the other group was switched to the fish oil diet. The degree of *n*-3 PUFA incorporation into the liver phosphatidylcholine was experimentally evaluated in one representative animal by weekly measuring the EPA + DHA:AA ratio. The highest value of this ratio was reached in animals fed the fish oil diet after 5 weeks. At this moment, EPA + DHA:AA ratios in animals fed the maize oil or fish oil diet were 0·2 and 3·7, respectively. The food consumption (4·0 (SD 1·7) g/d per animal) and body weight of the experimental animals were assessed twice per week from the beginning of the dietary treatment to the end of the experiment. At the end of the experiments, animals showed a similar increase (30·4 %) of body weight,

regardless of whether they were fed the maize oil or fish oil diet (data not shown).

Determination of lung colonisation of F10-SR cells transplanted in animals fed maize oil or fish oil diets

A F10-SR cell suspension (0·2 ml; 5×10^5 cells/ml Dulbecco's modified Eagle's medium) was injected into the lateral tail vein of experimental animals of the two groups fed their respective diets for 5 weeks. The animals were maintained at their respective diets until the end of experiment, i.e. 3 weeks after tumour cell injection. Animals were killed by cervical translocation under diethyl ether anaesthesia. Lung surface was inspected with the aid of a dissection microscope, and surface pulmonary colonies were counted, exsected from lung parenchyma and submitted to immunohistochemical analyses.

Gas-chromatographic analyses of fatty acids

Total lipids were extracted by the method of Folch *et al.*[38] from liver homogenates. Phosphatidylcholine was separated from the other lipid classes by HPLC following the conditions reported in a previous paper[7]. Phosphatidylcholine, solubilised in 0·2 ml benzene, was submitted to transmethylation with 5 % H_2SO_4 in methanol, at 80°C, overnight. The methyl-ester derivatives of fatty acids were analysed on a 30 m × 0·53 mm internal diameter wide-bore capillary column (Megabore W DB-23, 50 % cyanopropyl polysiloxan; J & W Scientific, Folsom, CA, USA), mounted in a PerkinElmer gas chromatograph equipped with a hydrogen flame detector (PerkinElmer, Waltham, MA, USA). Fatty acid composition was determined from the total integrated area in the chromatogram. Percentages of EPA, DHA and AA were used to calculate EPA + DHA:AA ratios in liver phosphatidylcholine.

Immunohistochemistry

Immunoreactivities of PCNA, bax/bcl-2, VEGF and vWF were determined in 5 μm thick sections of formalin-fixed paraffin-embedded metastatic colonies, according to a procedure described in a previous paper[7]. Monoclonal antibodies against PCNA, bax/bcl-2 and VEGF were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the polyclonal antibody against mouse vWF was supplied by Dako (Glostrup, Denmark). Negative controls were represented by consecutive tissue sections submitted to the immunohistochemical procedure in which the primary antibodies were omitted.

The immunoreactivities of PCNA, bax, bcl-2 and VEGF were scored following a semi-quantitative procedure that graded the percentages of reactive cells as follows: +, focal (less than 10 %); ++, moderate (10 to 50 %); +++, strong staining (>50 % stained cells); −, absent. Microvessel densities in the pulmonary colonies grown in animals fed the maize oil or fish oil diets were derived from the number of vWF-positive cells/microscopic field.

Detection of apoptotic nuclei

In order to confirm apoptotic activity in the pulmonary colonies developed in animals fed the maize oil or fish oil diets, DNA fragments in apoptotic nuclei were visualised according

to the method of Gavrieli *et al.* [39]. This method is based on the reaction, catalysed by terminal deoxynucleotidyl transferase, of 3′-OH ends of DNA fragments with biotin-labelled deoxynucleotides (Oncogene Research Products, Cambridge, MA, USA). The biotinylated nucleotides were complexed with a streptavidin–horseradish peroxidase conjugate, followed by visualisation with diaminobenzidine chromogen. Sections were counterstained with a 0·3 % methyl green solution and mounted on slides in a xylene mounting medium.

Statistical analysis

The Mann–Whitney test was used to determine the statistical significance of the differences between animals fed the maize oil diet and animals fed the fish oil diet in lung colonisation and microvessel densities.

Results

As shown in Fig. 1, F10-SR cells reproduced a lower number of metastatic colonies in the lungs of mice fed the fish oil diet, as compared with mice fed the maize oil diet. Regardless of the dietary regimen, PCNA (Figs. 2 (a) and (b)) was highly expressed in the pulmonary colonies developed in animals transplanted with F10-SR cells. Bax was moderately immunoreactive in the pulmonary colonies grown in animals fed the maize oil diet (Fig. 2 (c)), but highly expressed in the pulmonary colonies grown in animals fed the fish oil diet (Fig. 2 (d)). Regardless of the dietary regimen, pulmonary colonies did not express any reactivity for bcl-2 (see insert). Apoptotic nuclei, visualised by specific labelling of DNA fragments, were detectable only in metastatic colonies grown in animals fed the fish oil diet, as indicated by arrows in Fig. 2 (f).

As shown in Fig. 3, VEGF immunoreactivity was rather weak in the pulmonary colonies developed in animals fed the maize oil diet (Fig. 3 (a)), but fairly strong in colonies grown in animals fed the fish oil diet (Fig. 3 (b)). In these latter, however, there was a reduction of microvessel density, as determined by the visualisation of endothelial-associated vWF (Fig. 3 (d)).
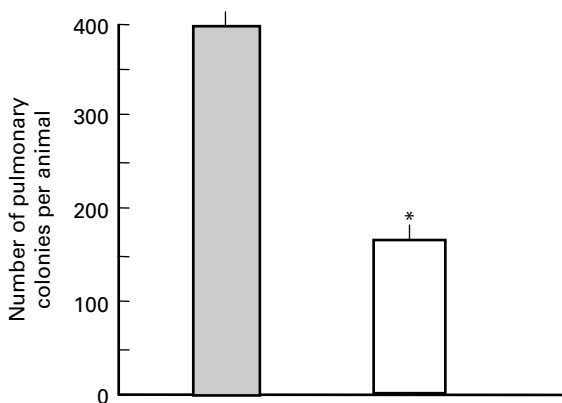

Fig. 1. Lung colonisation of F10-SR cells in animals fed maize oil (□) or fish oil (□) diets. Values are the means of number of lung colonies per animal (*n* 5), with standard errors represented by vertical bars. *Mean value was significantly different from that of the animals fed the maize oil diet (*P* = 0·004).

Discussion

In the present study, we demonstrated that the development of experimental metastases from a highly metastatic murine melanoma line, the F10-SR cells, was inhibited in animals fed a diet containing 5 % fish oil, a concentration much lower than that used in other studies [40]. In contrast with the present results, Salem *et al.* [28] found an accentuated growth of primary tumour and an increased development of lung metastases from B16 melanoma cells in mice treated with subcutaneous administration of fish oil before and after subcutaneous transplantation of tumour cells. The discrepancy between our and Salem's results might be related to the different experimental protocols used in the respective studies. An antimetastatic activity of *n*-3 PUFA was also found by Reich *et al.* [24] who obtained a reduction of lung colonisation of B16-F10 melanoma cells which had been previously enriched *in vitro* with EPA, an observation also made in our laboratory (A Mannini and S Ruggieri, unpublished results). Moreover, Iigo *et al.* [25] found an inhibition of lung colonisation in animals injected with colon carcinoma cells which had been previously submitted to a serial transplantation into mice fed an *n*-3 PUFA-rich diet. Reich's and Iigo's observations would suggest that the inhibitory activity of dietary *n*-3 PUFA on metastatic growth is mediated by their incorporation into tumour cells, although a contribution of lipid changes in host cells may not be ruled out.

In order to investigate which biological activities mediate the inhibition of lung colonisation of F10-SR cells in animals fed a fish oil diet and injected with F10-SR cells, we examined immunohistochemically PCNA, bax/bcl-2 and VEGF/vWF, as markers of growth, apoptosis and vascularisation, respectively. This investigation revealed that the reduced lung colonisation of melanoma cells in animals fed a fish oil diet is associated with an increased apoptotic activity, as shown by the enhancement of immunoreactivity of bax combined with a visualisation of nuclear DNA fragments in pulmonary colonies. This finding is in agreement with the positive correlation between the consumption of fish oil diets and apoptotic activity reported in several studies [40–42]. The unchanged PCNA immunoreactivity in the lung colonies developed in animals fed a fish oil diet suggests that the antimetastatic effect of dietary *n*-3 PUFA is not mediated by an inhibition of tumour cell proliferation, a finding in contrast with the selective antiproliferative effects of *n*-3 PUFA observed in different systems of tumour cells grown in tissue culture [43–47]. However, the artificial conditions produced in *in vitro* studies, not comparable with the complex *in vivo* tumour microenvironment, limit the significance of the data derived from *in vitro* studies regarding the influence of specific lipid components, including PUFA, on tumour growth (for a review, see Diggle [48]).

The diminution of microvessel density found in pulmonary colonies developed from F10-SR cells in animals fed a fish oil diet might contribute to the antimetastatic effect of dietary *n*-3 PUFA. This observation is supported by the finding that the reduction of murine mammary tumour growth by dietary *n*-3 PUFA is mediated by an inhibition of angiogenesis [49,50]. The association of a reduced microvessel density with a high VEGF immunoreactivity in pulmonary colonies developed in animals fed a fish oil diet is quite an unexpected
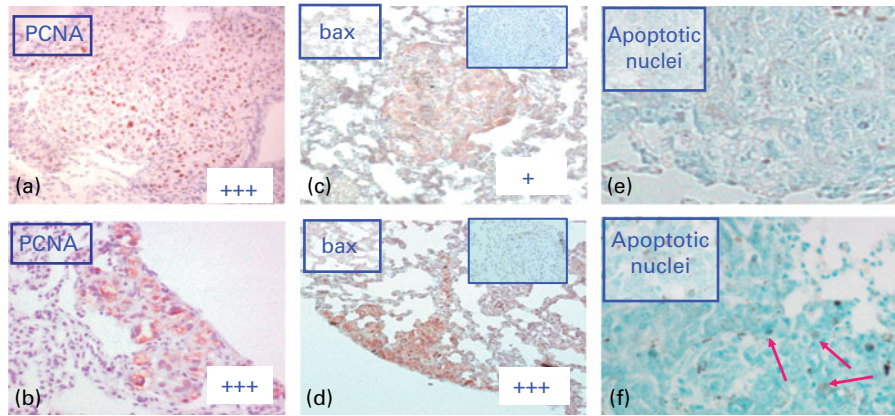
Fig. 2. Immunoreactivities of proliferating cell nuclear antigen (PCNA), bax and bcl-2 (see inserts in c and d) and visualisation of apoptotic nuclei (←) in pulmonary colonies developed from F10-SR cells in animals fed maize oil (a, c, e) or fish oil (b, d, f) diets. The immunoreactivity of each marker was evaluated as reported in Materials and methods. +, Focal staining (less than 10 %); +++, strong staining (>50 % stained cells).

finding. However, it is possible that *n*-3 PUFA inhibit vascularisation by counteracting the stimulatory activity of VEGF on vascular endothelia. Indeed, the competition between dietary *n*-3 PUFA and *n*-6 PUFA inhibits the synthesis of PGE2[51,52], an essential factor in angiogenesis[53,54]. Moreover, Salcedo *et al.*[54] showed that the inhibition of PGE2 synthesis abrogates the VEGF-mediated expression of CXCR4, a key receptor in angiogenesis.

In conclusion, the data derived from the present study point out the possibility that even limited amounts of *n*-3 PUFA in the diet are able to inhibit the reproduction of experimental metastases from F10-SR cells. The reduced formation of metastases from F10-SR cells transplanted into animals fed an *n*-3 PUFA-rich diet might be the final step of a series of phenomena, consisting of: a decreased generation of PGE2– reduced microvessel density – hypoxia – induction of apoptosis. This sequence, however, does not rule out the possibility that promotion of apoptosis and inhibition of angiogenesis represent two independent responses to the accumulation of *n*-3 PUFA.
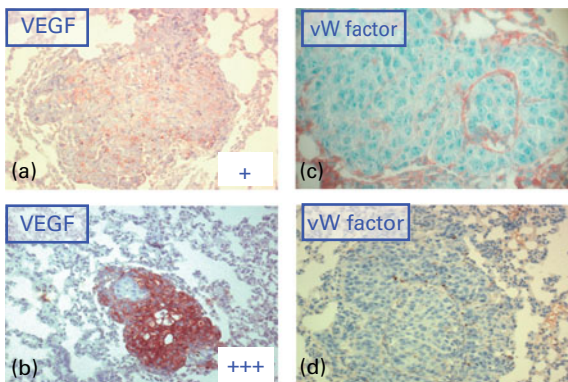

Fig. 3. Immunoreactivities of vascular endothelial growth factor (VEGF) and endothelial von Willebrand factor (vWF) in pulmonary colonies developed from F10-SR cells in animals fed maize oil (a, c) or fish oil (b, d) diets. The immunoreactivity of each marker was evaluated as reported in Materials and methods. +, Focal staining (less than 10 %); +++, strong staining (>50 % stained cells). The mean number of vWF-positive cells/microscopic field was (*n* 10) 41·6 (sem 3·6) in (c) and 22·0 (sem 1·9) in (d) (*P*<0·05).

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