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Promotion of colon cancer metastases in rat liver by fish oil diet is not due to reduced stroma formation

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Abstract

Recently, it was demonstrated that dietary Ω-3 polyunsaturated fatty acids (PUFAs) induce 10-fold more metastases in number and 1000-fold in volume in an animal model of colon cancer metastasis in rat liver. It was observed that tumors of rats on a fish oil diet lacked peritumoral stroma unlike tumors in livers of rats on a low fat diet or a diet containing Ω-6 PUFAs. In the present study, only one-third of the tumors in livers of rats on Ω-3 PUFA diet contained peritumoral stroma, whereas peritumoral stroma was present in 87% of the tumors in livers of rats on low fat diet. To explain these findings, we tested the hypothesis that fish oil exerts a direct inhibiting effect on the formation of extracellular matrix in tumor stroma as a consequence of blocking transformation of fat storing cells into myofibroblasts. It was found with immunohistochemical analysis of desmin as marker for fat storing cells and α-smooth muscle actin as marker for myofibroblasts that numbers of myofibroblasts were higher in tumors containing intratumoral stroma only than in tumors containing both peritumoral and intratumoral stroma. As most of the tumors in fish oil-treated rats contained intratumoral stroma only, this suggests that transformation of fat storing cells into myofibroblasts was highest in tumor stroma of fish oil-treated rats. Therefore, it is unlikely that the lack of stroma around tumors in fish oil-treated rats is due to inhibition of transformation of fat storing cells into myofibroblasts, but lack of peritumoral stroma is rather a consequence of rapid development of tumors in livers of fish oil-treated rats.

Introduction

Dietary fish oils have an inhibitory effect on development of primary tumors [1]. However, fish oil diet enriched with Ω-3 polyunsaturated fatty acids (PUFAs) has a profound stimulating effect on the development of experimentally induced colon carcinoma metastasis in rat liver [2]. It was shown that fish oil induced 10-fold more metastases in number and 1000-fold in volume, in comparison with metastases grown in livers of rats on a low fat diet. This effect did not seem to be mediated by alterations in the immune system since immunocompetent cells in normal liver parenchyma were not significantly affected by the diet. At three weeks after administration of colon cancer cells, numbers of immunocompetent cells were even higher in livers of rats fed with fish oil diet [2]. A striking finding was that the amount of tumor-associated stroma was strongly decreased in livers of fish oil-treated rats.

Tumor stroma is a dynamic tissue structure that is produced by host cells in response to cancer cells [3, 4]. It consists of extracellular matrix and cells such as fibroblasts, which are responsible for the deposition of the extracellular matrix, inflammatory cells and endothelial cells which play a role in angiogenesis. Stroma plays a major role in tumor development since it forms both the lifeline for tumors providing vessels and a barrier limiting tumor expansion [4–8].

Extracellular matrix in stroma of both hepatocellular carcinomas and metastatic colon carcinomas in human liver is formed by myofibroblasts which are largely transformed hepatic fat storing cells [9, 10]. Fat storing cells, also called Ito cells or stellate cells, are located in the space of Disse in sinusoids of the liver. They contain vitamin A in lipid droplets and are involved in uptake, storage and release of retinoids and normal turnover of extracellular matrix in liver [11, 12]. Under pathological conditions, fat storing cells are activated and transformed into myofibroblast-like cells [11]. These cells are highly proliferative and produce large

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amounts of collageneous and non-collageneous extracellular matrix components [10, 13, 14]. Transformation of fat storing cells into myofibroblasts is accompanied by loss of lipid droplets and an increased content of α-smooth muscle actin [9]. Transformation is promoted by cytokines such as transforming growth factor β and plateled derived growth factor (PDGF) which are secreted by activated Kupffer cells, macrophages and platelets [11, 15] and also by cancer cells [16, 17]. There are indications that Ω-3 PUFAs inhibit production of PDGF [18, 19]. In the present study, we tested the hypothesis that promotion of metastasis by fish oil is due to inhibition of transformation of fat storing cells into myofibroblasts and thus lack of stroma formation. We investigated these processes in an *in vivo* rat model of colon cancer metastasis in liver.

Materials and methods

Animals

Adult male and female Wag-Rij rats (3 months old at the start of the experiments; Broekman, Someren, The Netherlands) were maintained for 2 weeks under constant environmental conditions with free access to food and water. Afterwards, the animals were divided into 3 diet groups of 23 rats each. One group was kept on low fat diet, one on fish oil diet and one on Ω-6 PUFAs-containing safflower oil diet. Each group contained 17 female and 6 male rats. The animals were kept for 3 weeks on the respective diets before administration of the cancer cells. The animals were housed individually at the Academic Medical Center animal facility in accordance with the guidelines for animal care of the University of Amsterdam. All animals were weighed once every 3 days during the entire experiment.

Diets

Diets were prepared by Hope Farms (Woerden, The Netherlands). Fish oil (Pronova Biocare, Sandefjord, Norge) and safflower oil (Sigma, St. Louis, Missouri) were added each day to the food immediately before it was given to the animals. Fish oil and safflower oil were kept at 4 °C under nitrogen to avoid autooxidation of PUFAs. Vitamin E levels in the food were kept at 35 mg per kg low fat diet [5% (v/w) soybean oil], at 17.5 g per kg Ω-3 PUFA-containing diet and at 75 mg per kg Ω-6 PUFA-containing diet [20% (v/w) fish oil and safflower oil, respectively]. Other vitamins, minerals, essential fats and proteins were present in sufficiently high concentrations in the food to provide adequate growth [1]. Complete composition of the diets is reported by Van Noorden [20]. Each rat received 30 g food per day.

Cancer cells

An established colon carcinoma cell line (CC531) was obtained from a moderately differentiated and weakly immunogenic colon adenocarcinoma after experimental induction in Wag-Rij rats by treatment with 1,2′-dimethylhydrazine [21]. Cells were cultured at 37 °C as monolayers in Dulbecco's modified Eagle's medium (ICN Biomedicals, Irvine, Ayrshire, UK) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 IU penicillin/ml and 100 mg streptomycin/ml. Cells were washed with phosphate buffered saline (PBS) and after detachment with 0.05% trypsin in PBS and centrifugation (250 *g*, room temperature, 10 min), single cell suspensions were obtained with a viability of at least 95%.

Treatment

After 3 weeks of diet, small midline incisions were made in the abdominal walls of the rats and a suspension containing 1×10^6 cancer cells in 50 μl PBS was injected into the portal veins under sodium pentobarbital anaesthesia using a 27-gauge needle as described before in detail [22, 23]. Portal veins were closed by treatment of the incision with an aqueous solution of thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) using a cotton bud. The site of injection was covered by Spongostan (Medical Workshop, Groningen, The Netherlands) to prevent peritoneal seeding. The rats were kept on the respective diets until they were sacrificed. As controls, 3 female Wag-Rij rats were used for each diet group. These animals did not receive cancer cells. At 1 and 3 weeks after operation, 7 female and 3 male rats of each group were sacrificed by an overdose of sodium pentobarbital and livers were immediately removed, separated into lobes and cut in small pieces (5 mm thick) according to a rigid scheme [24]. The origin of all pieces of tissue was registered. The pieces were immediately frozen in liquid nitrogen and stored at −80 °C until used. All experiments were carried out in accordance with the guidelines for animal care of the University of Amsterdam.

Immunohistochemistry

Immunohistochemical procedures were carried out on 8 μm thick cryostat sections according to Polak and Van Noorden [25]. Sections were dried overnight and fixed in acetone for 10 min at −20 °C. Endogenous peroxidase activity was never present in this study and therefore, blocking procedures were omitted.

To demonstrate colon cancer cells, sections were incubated with the lectin Ulex europaeus agglutinin-I (UEA-I; Dako, Glostrup, Denmark). The lectin binding sites were detected with a monoclonal antibody against UEA-I as described before [2, 26].

For the detection of fat storing cells and myofibroblasts, respectively, serial sections were incubated with a monoclonal antibody against desmin (Sanbio, Uden, The Netherlands) diluted 1:50 in the presence of 1% bovine serum albumine (BSA) in PBS for 90 min [27] and a monoclonal antibody against α-smooth muscle actin (Dako) diluted 1:200 in the presence of 1% BSA in PBS for 2 h [28]. Sections were then incubated for 1 h in the presence of rabbit anti-mouse IgG conjugated with horseradish peroxidase (Dako) diluted 1:20 (for desmin) and 1:100 (for α-smooth muscle actin) in the presence of 1% BSA and 5% normal

Table 1. Distribution patterns of stroma in metastases in livers of rats fed with different diets at 3 weeks after administration of colon cancer cells. Numbers of metastases containing different types of stroma (peritumoral, intratumoral, or both) are expressed as percentages (%) of the total number of metastases investigated.

Diet	Rats (n)	Metastases (n)	Peritumoral stroma only (%)	Intratumoral stroma only (%)	Peritumoral and intratumoral stroma (%)
Low fat	6	15	0	13.3	86.7
Safflower oil	4	57[b]	17.5	47.4	35.1
Fish oil	4	129[a,c]	7.0	69.8	23.2

[a]Three metastases did not contain stroma
[b]One metastasis contained only partially peritumoral stroma
[c]Five metastases contained only partially peritumoral stroma

Table 2. Numbers of desmin-positive cells in liver parenchyma of rats on different diets before and at 3 weeks after administration of CC531 colon cancer cells. Data are expressed as mean numbers of cells/mm^2 of tissue $\pm$ SD

Diet	Before administration	At 3 weeks after administration
Low fat	6.7 ± 2.4	1.4 ± 1.7[a]
Safflower oil	9.3 ± 2.1	2.3 ± 3.1[a]
Fish oil	5.0 ± 3.2	17.6 ± 16.2[a,b]

[a]Significantly different from numbers of cells in livers of rats before administration of CC531 colon cancer cells.
[b]Significantly different from numbers of cells in other diet groups.

rat serum in PBS. Between the different incubation steps, sections were rinsed 3 times in PBS. Peroxidase activity was visualized using a medium containing 50 mg 3,3′-diaminobenzidine (Fluka, Buchs, Switzerland) dissolved in 100 ml 50 mM Tris-HCl buffer, pH 7.6, and hydrogen peroxide in a final concentration of 0.01% (v/v). The nuclei were counterstained with haematoxylin. Controls were performed in the absence of the primary antibody.

Localization of collagen

Staining of collagen was performed using Sirius Red F3BA in saturated picric acid as described before [29].

Cell counting

Numbers of desmin-positive cells in liver parenchyma of rats fed with different diets before and at three weeks after administration of the cancer cells were counted in 14 sections of different liver lobes of 5 animals per group. In each section, 3 periportal and 3 pericentral areas were randomly selected. Desmin-positive cells per unit area were quantified in a standardized microscopic field using a Zeiss microscope and a Neofluor objective $\times 40$. Data were expressed as the mean numbers of cells $\pm$ SD in liver parenchyma. Significance of differences was determined with ANOVA; $P \leq 0.05$ was considered significant.

Results

Stroma in and around tumors

At 1 week after administration of colon cancer cells, metastases were found in all livers irrespective the diets of the rats. All tumors contained colon cancer cells and inflammatory cells, but no extracellular matrix. Therefore, these livers were not analysed in detail with respect to transformation of fat storing cells.

At 3 weeks after administration of colon cancer cells livers contained metastases consisting of cancer cells, inflammatory cells and extracellular matrix. Distribution patterns of tumor-associated stroma differed distinctly in a diet-dependent manner. Metastases were classified and defined on the basis of the presence of peritumoral or intratumoral stroma or both (Table 1). Peritumoral stroma was defined as stroma surrounding cancer cells, thus separating cancer cells from liver parenchyma, whereas intratumoral stroma was localized in between cancer cells. Examples of both types of stroma are shown in Figure 1. The larger part of tumors in the control group (87%) contained both peritumoral and intratumoral stroma, whereas the remainder (13%) contained intratumoral stroma only. In contrast, 23% of the tumors in the fish oil group contained both peritumoral and intratumoral stroma, whereas 70% of the tumors lacked peritumoral stroma. In the group of safflower oil-fed animals, the numbers of tumors lacking peritumoral stroma and containing both peritumoral and intratumoral stroma were rather similar (47% vs. 35%, respectively). Collagen was present at the basement membrane separating colon cancer cells from the stroma compartment in both types of metastases. Furthermore, strands of collagen were observed within peritumoral and intratumoral stroma of the tumors (Figures 1C and D).

Distribution of desmin-positive cells

In livers of rats without cancer, desmin-positive cells were observed in sinusoids in liver parenchyma, in portal tracts and around central veins (Figure 2A). Numbers of desmin-positive cells in liver parenchyma were similar in the 3 diet groups (Table 2). Three weeks after administration of colon cancer cells, numbers of desmin-positive cells in sinusoids of liver parenchyma were decreased in rats fed with low fat diet and safflower oil-containing diet as compared with animals without metastases, but were increased in rats fed with fish oil-containing diet (Table 2). Moreover, desmin-positive cells were present in peritumoral and intratumoral stroma of tumors in all diet groups (Figures 1E and F). Desmin-positive cells were rather homogeneously distributed over the different types of stroma and correlated with the presence of collagen (cf. Figures 1C and E, D and F).

Distribution of α-smooth muscle actin-positive cells

α-Smooth muscle actin-positive cells were found in connective tissue of portal tracts and vessel walls in animals without cancer and in animals with colon cancer metastases (Figure 2B). α-Smooth muscle actin-positive cells were also present in peritumoral and intratumoral stroma of tumors in all diet groups (Figures 1G and H). However,

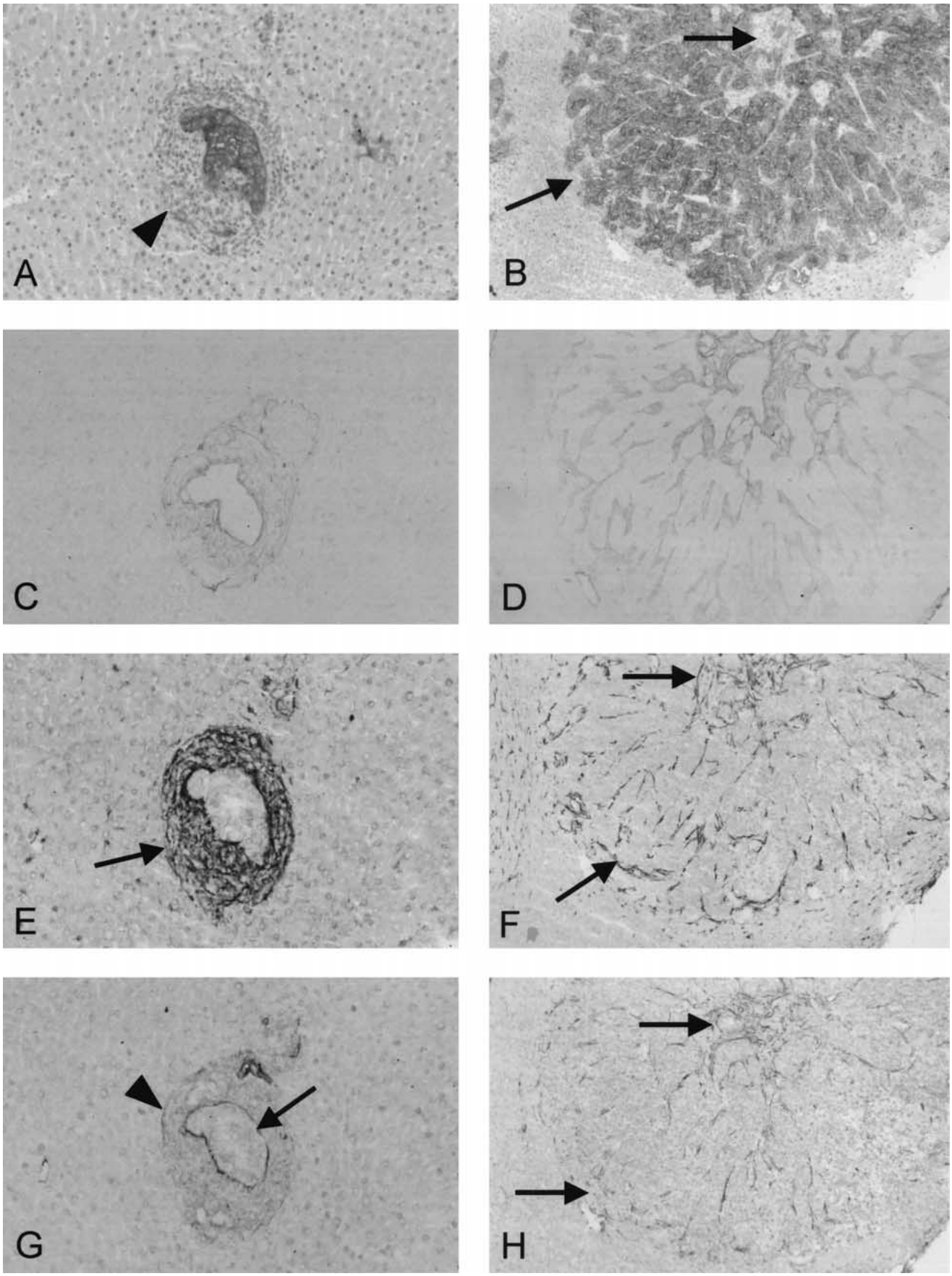

Figure 1. Metastases in livers of rats treated with low fat diet (A, C, E, G) and fish oil diet (B, D, F, H) at 3 weeks after administration of colon cancer cells into the vena portae and stained for lectin present in cancer cells (A, B), collagen (C, D), desmin (E, F) and α-smooth muscle actin (G, H). Intratumoral stroma is defined as stroma elements in between cancer cells (B, arrows), whereas peritumoral stroma is defined as stroma surrounding cancer cells, thus separating cancer cells from liver parenchyma (A, arrowhead). Collagen was present at the basement membrane of colon cancer cells in both types of metastases (C, D) and strands of collagen were found within peritumoral (C) and intratumoral stroma (D). Large numbers of desmin-positive cells were present in peritumoral and intratumoral stroma (E and F, arrows). α-Smooth muscle actin-positive cells were found mainly at the periphery of tumors (arrowhead) and surrounding cancer cells in the low fat diet group (G, arrow) and in intratumoral stroma in fish oil rats (H, arrows).

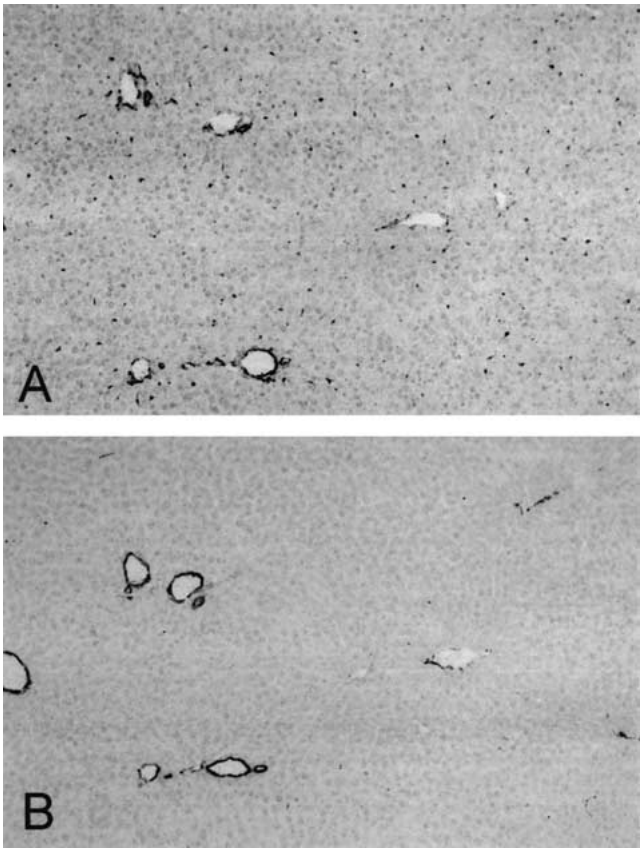

Figure 2. Distribution pattern of desmin-positive cells (A) and α-smooth muscle actin-positive cells (B) in liver parenchyma of rat treated with low fat diet. Desmin-positive cells were observed in sinusoids of liver parenchyma, in portal tracts and around central veins (A). α-Smooth muscle actin-positive cells were found in connective tissue of portal tracts and vessel walls (B).

there were distinct differences in the distribution patterns of α-smooth muscle actin-positive cells and desmin-positive cells in tumor-associated stroma in the 3 diet groups (Figures 1E–H). These differences were related with the type of stroma in the tumors rather than with the diet. In metastases with peritumoral and intratumoral stroma, α-smooth muscle actin-positive cells were mainly located at the edges of stroma, adjacent to cancer cells and liver cells (Figure 1G). In contrast, metastases with intratumoral stroma only contained α-smooth muscle actin-positive cells in all areas where desmin-positive cells and collagen were also found (cf. Figures 1D, F and H).

Discussion

We have shown that tumors grown in livers of rats fed with fish oil have less peritumoral stroma than tumors grown in rats fed with safflower oil-containing diet or low fat diet (Table 1). This lack of peritumoral stroma may be explained in two ways. First, fish oil inhibits tumor stroma formation directly and thus facilitates growth of the metastases. Second, fish oil stimulates growth of metastases directly and due to their rapid growth, formation of stroma is restricted. It has been reported that myofibroblasts transdifferentiated from fat storing cells are mainly responsible for formation of extracellular matrix in tumor stroma in liver [30]. We tested the hypothesis that fish oil exerts a direct inhibiting effect on the formation of extracellular matrix in stroma by affecting the transformation of fat storing cells into myofibroblasts. Fat storing cells and myofibroblasts were identified immunohistochemically by positivity for desmin and α-smooth muscle actin, respectively. The intermediate filament protein desmin is found in rat liver, both in fat storing cells and myofibroblast-like cells as well as in portal and capsular fibroblasts [31]. The myofibroblast-like phenotype is characterized by expression of α-smooth muscle actin, a protein that is also present in smooth muscle cells. Staining of collagen was used to confirm the presence of collagen in relation to (myo)fibroblasts.

First of all, we observed a significantly higher number of fat storing cells in liver parenchyma of fish oil-fed rats than in rats fed with the other diets at 3 weeks after administration of colon cancer cells. These differences were not observed when cancer cells were not given. Moreover, when metastases were present in the livers of rats fed with low fat and safflower-containing diets numbers of desmin-positive cells (fat storing cells) were significantly reduced in liver parenchyma. This loss of fat storing cells may implicate loss of vitamin A and lipid contents in livers, which may be related to induction of lipolysis by adenocarcinomas in adipocytes. Lipolysis was prevented by feeding with fish oil [32]. Therefore, Ω-3 PUFAs are currently used to prevent cachexia in cancer patients. We also compared positivity for desmin with positivity for α-smooth muscle actin in metastases in each diet group and we observed a similar distribution pattern of both cell types in metastases with intratumoral stroma only. Moreover, we found that areas in stroma containing high amounts of collagen always showed a high density of myofibroblasts. These data suggest that collagen in intratumoral stroma was always produced by myofibroblasts, irrespective the diet. This finding leads to the conclusion that transformation of fat storing cells into myofibroblasts was not inhibited by fish oil.

In metastases containing both peritumoral and intratumoral stroma which were mainly present in livers of low fat diet rats, we observed a similar distribution pattern of desmin-positive cells and α-smooth muscle actin-positive cells only at the periphery of the tumor. This indicates that transformation of fat storing cells into myofibroblasts occurrs at the edge of tumors. The observation that both in peritumoral and intratumoral stroma areas are positive for desmin and collagen and negative for α-smooth muscle actin suggests that fibroblasts and not fat storing cells transformed into myofibroblasts are involved in stroma formation in these areas. These findings suggest that the extracellular matrix of tumor stroma may have been produced partly by fibroblasts and partly by myofibroblasts. This is in contrast with observations of Terada et al. [9].

On the basis of our data, we like to conclude that other mechanisms than inhibition of transformation of fat storing cells into myofibroblasts are responsible for the diminished amounts of extracellular matrix around the metastases in fish

oil-treated rats. Dingemans et al. [7] have shown that small metastases ($< 400\ \mu$m) consisted of well-differentiated acini, fully surrounded by connective tissue that was derived from the portal tract. In larger metastases, this connective tissue was overgrown by tumor cells and acinar differentiation was lost. Griffini et al. [8] demonstrated, based on 3D reconstructions of colon cancer metastases, that all large metastases were in close contact with the Glisson capsule and that these tumors only contain intratumoral stroma. On the other hand, small metastases contained peritumoral stroma which was continuous with stroma in portal tracts. These findings give support to the conclusion that the developmental stage of tumors determines the presence or absence of peritumoral stroma. Recent studies in our group demonstrated that a more aggressive CC531 (CC531s) line gives rise to metastases in rat liver which always lack peritumoral stroma at 3 weeks after administration. Therefore, the promoting effect of fish oil on colon cancer metastases in rat liver may be due to a direct effect of fish oil on tumor growth. This stimulus may be caused by an enhanced uptake of purines by cancer cells treated with fish oil which are needed for proliferation as observed by Martin and Meckling-Gill [33]. Another explanation may be that fish oil causes downregulation of the immune system in rat liver. Although it has been reported before that the dramatic effect of Ω-3 PUFA on the growth of colon cancer metastases in the liver was not mediated via alterations of the immune system at later stages of metastasis [2], it cannot be excluded that early changes in the immune system play a decisive role in metastasis formation in fish oil-fed rats directly after administration of cancer cells. These possibilities need further investigation.

In conclusion, the dramatic stimulating effect of fish oil on the growth of colon cancer metastases in rat liver and the lack of peritumoral stroma in these metastases is not a consequence of a lowered transformation rate of fat storing cells into myofibroblasts that ultimately result in reduced deposition of tumor stroma.

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