

Sensitization by Dietary Docosahexaenoic Acid of Rat Mammary Carcinoma to Anthracycline: A Role for Tumor Vascularization

Séverine Colas,^{1,3} Karine Mahéo,¹ Fabrice Denis,¹ Caroline Goupille,¹ Claude Hoinard,¹ Pascal Champeroux,³ François Tranquart,² and Philippe Bougnoux¹

Abstract Purpose: To investigate whether dietary docosahexaenoic acid (DHA), a peroxidizable polyunsaturated ω -3 fatty acids, sensitizes rat mammary tumors to anthracyclines and whether its action interferes with tumor vascularization, a critical determinant of tumor growth.

Experimental Design: Female Sprague-Dawley rats were initiated by *N*-methylnitrosourea to develop mammary tumors and then assigned to a control group ($n = 18$), receiving a supplementation of palm oil, or to a DHA group ($n = 54$), supplemented with a microalgae-produced oil (DHASCO, 1.5 g/d). The DHA group was equally subdivided into three subgroups with addition of different amounts of α -tocopherol. Epirubicin was injected weekly during 6 weeks after the largest tumor reached 1.5 cm², and subsequent changes in the tumor surface were evaluated. Tumor vascularization was assessed by power Doppler sonography before and during chemotherapy.

Results: DHA and α -tocopherol were readily absorbed and incorporated into rat tissues. Epirubicin induced a 45% mammary tumor regression in the DHA-supplemented group, whereas no tumor regression was observed in the control group. In the DHA group, before chemotherapy was initiated, tumor vascular density was 43% lower than in the control group and remained lower during chemotherapy. Enhancement of epirubicin efficacy by DHA was abolished in a dose-dependent manner by α -tocopherol, and the same trend was observed for DHA-induced reduction in tumor vascular density.

Conclusions: Dietary DHA supplementation led to a reduction in tumor vascularization before the enhancement of any response to anthracyclines, suggesting that DHA chemosensitizes mammary tumors through an inhibition of the host vascular response to the tumor.

In breast cancer, the most important determinant of tumor response to treatment is tumor cell sensitivity to cytotoxic agents that are given during initial treatment, either as induction chemotherapy or as adjuvant to locoregional therapy. Occurrence of resistance to chemotherapy is a major obstacle to successful treatment. Thus, factors that increase sensitivity of tumors to anticancer drugs contribute to improvement of patient survival. Several studies highlighted the fact that n-3 and n-6 polyunsaturated fatty acids (PUFA)

enhance the sensitivity of tumor cells to chemotherapy *in vitro* (1–3), suggesting an involvement of the degree of fatty acid unsaturation. Dietary n-3 PUFA also chemosensitized tumors *in vivo* (4–7). All these studies were carried out with dietary fish oil; therefore, the effects observed could not be ascribed on any specific n-3 PUFA. A study carried out in patients presenting with breast cancer investigated the relation between adipose tissue docosahexaenoic acid (DHA) level, taken as an indicator of past dietary DHA intake, and tumor sensitivity to neoadjuvant chemotherapy. An association between DHA level in breast adipose tissue and response of the breast tumor to anthracycline-containing chemotherapy has been reported (8). This suggests that n-3 PUFA-enhanced anticancer drug activity observed with fish oil may also apply to human breast cancer and that DHA may be an appropriate candidate for this activity.

Among PUFA, DHA was found to be the most potent fatty acid to enhance doxorubicin efficacy in breast cancer cell lines (3). Several anticancer agents, including anthracyclines, generate the production of reactive oxygen species (ROS) in cancer cells (9). DHA, with six double bonds, is very prone to oxidation. Actually, we identified an amplification of the oxidative stress and subsequent lipoperoxidation by DHA as a potential mechanism accounting for the sensitization of breast cancer cells to doxorubicin (10). Moreover, these effects of n-3 PUFA were found to be suppressed by the presence of

Authors' Affiliations: ¹Institut National de la Santé et de la Recherche Médicale, E0211 Nutrition Croissance et Cancer, Tours; IFR 135, Univ. Tours, Tours; ²Institut National de la Santé et de la Recherche Médicale, U619, Tours; IFR 135, Univ. Tours, Tours; and ³Centre de Recherches Biologiques, Baugy, France
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Requests for reprints: Philippe Bougnoux, Institut National de la Santé et de la Recherche Médicale E0211, CHU Bretonneau, 2 bis Boulevard Tonnellé, F-37044 Tours, France. Phone: 33-247-366179; Fax: 33-247-478065 or 33-247-366226; E-mail: bougnoux@med.univ-tours.fr.

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antioxidant molecules (3), and *in vivo*, in the presence of fish oil, dietary α -tocopherol decreased the efficacy of chemotherapy on mammary tumors (11). Similarly, the efficacy of radiation therapy, which generates ROS, was enhanced by n-3 PUFA both *in vitro* (12) or *in vivo* in mammary tumors (13) or in head and neck tumors (14). Therefore, the oxidative status, as well as the oxygen availability to the tumor, seems to be crucial determinants of its chemosensitivity or radiation sensitivity, stressing a role for tumor vascularization.

Changes in tumor vascularization may account for n-3 PUFA-induced effects on tumor. Vascular changes associated with anticancer treatments, such as radiation therapy or chemotherapy, are thought to be pivotal in subsequent tumor volume decrease or progression (15). Change in tumor vasculature has been reported to occur even before tumor regression, as suggested by the temporal changes in tumor blood flow and histologic blood vessel quantification reported in animal tumor systems (16) and in humans (17).

Thus, there is a need to determine *in vivo* whether the effects of fish oil on tumor sensitivity to anthracyclines can be ascribed to a defined component, such as DHA. To investigate whether dietary DHA enhances tumor sensitivity to anthracyclines and whether tumor vascularization is involved, we used the model of *N*-methylnitrosourea (NMU)-induced rat mammary tumors used previously to evaluate the action of fish oil (11). In this experimental system, autochthonous tumors develop from mammary glands (18) and are locally invasive adenocarcinoma with all constituents of tumor stroma, including neovessels. We carried out a dietary intervention with a DHA-enriched oil, examined tumor response to epirubicin according to diet, and quantified early tumor vascular changes by power Doppler sonography. Moreover, to determine if α -tocopherol inhibited these effects of DHA through a mechanism linked to tumor vascularization, we added two nutritional groups with two different doses of the antioxidant, α -tocopherol, and studied the subsequent tumor response and vascularization.

Materials and Methods

Animals and experimental carcinogenesis. A total number of 72 pathogen-free female Sprague-Dawley rats were purchased from Harlan (Gannat, France) when they were 40 days of age. Care of these animals was in accordance with institution guidelines. They were housed three per cage and maintained at constant temperature (22°C) and humidity with a 12-hour light/dark cycle. At 48 days of age (time of the maturation of their mammary glands), rats received a single dose of NMU (Sigma, Saint Quentin-Fallavier, France) by a s.c. injection (25 mg/kg body weight) to initiate mammary tumorigenesis as described previously (18, 19).

Diets. During the acclimatization period, rats received a standard diet (Harlan Teklad TRM rat/mouse diet, quality controlled). Two days after induction (i.e., NMU injection) up to the end of the experience, all rats received a diet (obtained from Institute for Agricultural and Food Research, Jouy-en-Josas, France) containing casein (22 g/100 g), methionine (0.16 g/100 g), cornstarch (37.3 g/100 g), sugar (18.7 g/100 g), cellulose (2 g/100 g), minerals (4 g/100 g), and vitamins A, D₃, K₁, B₁, B₅₋₉, B₁₂, and C (1 g/100 g). To this diet was added 7% (w/w) of a mixture of peanut and rapeseed oils (2/3 and 1/3, w/w, respectively). This basal diet was supplemented with 8% (w/w) additional lipids: control group was supplemented with palm oil (purchased from Société Industrielle des Oléagineux, St Laurent Blangy, France), and DHA groups were supplemented with DHASCO (graciously provided by Martek Biosciences Corp., Columbia, MD). The type and proportion of these oils were established in such a way that the final fatty acid composition did not differ markedly between groups, except for DHA, and any essential fatty acids deficiencies, such as linoleic acid (18:2n-6) or α -linolenic acid (18:3n-3), would be prevented (Table 1). Diets were prepared once weekly and refrigerated at +4°C until feeding. Rats were fed *ad libitum*, with 25 g/d/rat of food available. As the DHASCO contained ~45% of DHA, DHA intake was ~0.7 g daily. The DHA group was divided into three subgroups, which differed by their supply in α -tocopherol acetate: no addition of α -tocopherol acetate, 100 IU/kg, or 500 IU/kg. Basal level of α -tocopherol was 37 IU/kg in the palm group and 29 IU/kg in the DHA group.

Chemotherapy. Epirubicin (Pharmacia, Saint-Quentin-en-Yveline, France), which is the anthracycline used currently in breast cancer, was selected as the chemotherapeutic agent. Epirubicin was freshly prepared in saline and given as a weekly injection at a dose of 2.5 mg/kg

Table 1. Fatty acid composition of the oils and of the final oil mixture used in the control or DHA-supplemented diets

Fatty acids (% area)	Oils (%)				Diets (%)	
	Peanut	Rapeseed	Palm	DHASCO	Control	DHA supplemented
Saturates						
14:0	0.03	0.04	1.05	14.3	0.6	7.6
16:0	10.0	4.6	43.8	13.1	27.2	10.8
18:0	3.9	1.7	4.45	0.6	3.9	1.8
Total*	20.2	7.5	52.3	33.1	35.3	25.1
Monounsaturates						
18:1n-9c	56.8	59.4	37.4	17.8	46.9	36.4
Total†	58.2	61.8	37.8	18.0	47.9	37.3
n-6 PUFA						
18:2n-6c	20.7	20.9	9.2	1.3	14.6	10.4
Total‡	20.8	20.9	9.3	1.3	14.7	10.4
n-3 PUFA						
18:3n-3	0.1	8.8	0.2	<0.1	1.5	1.4
22:6n-3	0.1	<0.1	<0.1	44.8	<0.1	23.9
Total	0.2	8.8	0.2	45.1	1.5	25.5

*Including 20:0, 22:0, and 24:0.

†Including 16:1, 20:1, 22:1, and 24:1.

‡Including 18:3n-6.

by i.p. route during 6 weeks. Injections were initiated when the surface of the largest mammary tumor reached 1.5 cm². This size has been defined in previous studies as the best size for observing variations in tumor area during treatment. Epirubicin dose was chosen to induce a stabilization of tumor growth in the control group. From NMU injection to the beginning of chemotherapy, the time period required for the largest tumor to reach 1.5 cm² did not significantly differ among dietary groups: from 13.9 ± 1.6 weeks (mean + SE) in the control group to 12.5 ± 1.0 in the DHA group; This time was 11.5 ± 1.1 in the DHA + 100 IU vitamin E and 13.5 ± 1.3 in the DHA + 500 IU vitamin E ($P = 0.653$, Kruskal-Wallis one-way ANOVA).

Assessment of tumor chemosensitization. Four weeks after NMU induction, rats had a weekly mammary glands examination to determine the latency, location, incidence, and multiplicity of NMU-induced tumors. Tumor size was determined by measuring the two largest diameters with a caliper and tumor area was calculated as the product of the two measures according to the method recommended for human tumors (20). Tumor size change was calculated as the ratio between the difference in tumor size over a defined period divided by the initial tumor size, according to the formula: tumor size change = [(tumor area_{day x} - tumor area_{day 0}) / tumor area_{day 0}] × 100, where tumor area_{day 0} was 1.5 cm².

Tumor size was assessed at time points throughout the chemotherapy period and for one additional week. Tumor shrinkage was defined as a negative tumor size change. We refer to chemosensitization as increased tumor shrinkage for an identical dose of epirubicin.

Assessment of tumor vascularization. Gray scale and power Doppler sonography were done under anesthesia as already described (21). The duration of ultrasonography examination was limited to 30 minutes to prevent anesthesia-induced hypothermia. Tumors were scanned with a 7- to 10-MHz linear probe (LA 523) using a Technos scanner (Esaote, Genoa, Italy). Tumor compression was kept to a minimum, shown by the persistence of gel between the probe and the tumor on the monitor to avoid tumor flow changes. Gain and velocity scale were the same throughout the experiment for all tumors. The five best power Doppler sonography images of the tumor corresponding to the subjectively determined highest Doppler signal were obtained from different imaging planes. Images were stored digitally and then transferred to a computer for further quantitative analysis of vascularization. Measurements were done before chemotherapy and at midpoint of the chemotherapy period. The Power Doppler Index (PDI) of tumor vascularization was quantified using a dedicated software for color pixel quantification developed from a Matlab program. This software allows determination of the number of colored pixels in a manually drawn region of interest corresponding to the whole tumor, regardless of signal intensity. PDI was calculated as relative number of pixels in the ultrasound image displaying a power Doppler signal (i.e., the number of colored pixels/number of pixels in the region of interest). The mean PDI was calculated for each tumor using the mean value of five stored images of that tumor.

Tissue fatty acids analysis. At the end of the experiment, animals were humanely sacrificed and necropsied. Adipose, liver, and tumor tissues were harvested, rinsed in saline, and immediately frozen and kept in liquid nitrogen for biochemical analysis. A blood sample was taken and centrifuged to separate the serum. Serum was kept at -80°C for biochemical analyses. Eight rats were randomly selected in each dietary group to provide tissues for fatty acid determination. Determination of the fatty acid content of the adipose and tumor tissues was done as described previously (22). After lipids were extracted (23), triglycerides and phospholipids were purified by preparative TLC. Fatty acids were methylated with boron trifluoride and analyzed by capillary gas chromatography using a 50-m polar column and an on-column injector (24).

Tissue α -tocopherol analysis. Total lipids were extracted from liver, serum, adipose, and tumor tissues according to the Folch extraction procedure (23). α -Tocopherol was directly extracted with hexane without prior saponification (25). Samples were analyzed by high-pressure liquid

chromatography (25). This system consisted of an 80- μ L injection loop; solvent was methanol and was pumped at 1 mL/min through a reverse-phase octadecyl-bonded silica column (Spherisorb ODS2, Waters, Saint-Quentin-en-Yveline, France). Detection of tocopherols was done with a fluorometer with excitation wavelength 290 nm and detection at 334 nm, coupled with a SP4270 integrator (Spectra-Physics, San Jose, CA). Separated compounds were quantified by comparison of their peak areas with that of δ -tocopherol used as an internal standard.

Vascular endothelial growth factor quantification. Level of vascular endothelial growth factor (VEGF)-A was measured in serum and tumor extracts using a rat VEGF ELISA kit (RayBio, Norcross, GA) according to the manufacturer's instructions. These measures were carried out at the end of the chemotherapy period, in samples obtained during autopsy. For tumor extracts, a piece of tissue was minced in homogenizing buffer (provided with kit) with an UltraTurrax homogenizer (IKA Labortechnik, Staufen, Germany). Homogenized samples were centrifuged at 10,000 × g at 4°C for 15 minutes, and the supernatants were stored at -80°C. Protein concentration of the lysate was determined using a bicinchoninic acid protein kit (Pierce, Rockford, IL). Tumor VEGF was quantified after lysate dilution to ~500 μ g/mL.

Statistical analysis. Tumor size regression was expressed as mean ± SE. Repeated measured ANOVA with grouping factor (time) was used to compare dietary groups as a function of diet and time after determination that the variables followed a normal distribution. To determine whether differences in the mean values for fatty acids, α -tocopherol, and vascularization analyses were significant, Kruskal-Wallis and Mann-Whitney tests were used. To isolate those dietary groups that differ from the others, a Dunn's test was used. The level of statistical significance was set at $P < 0.05$.

Results

Fatty acids composition of adipose and tumor tissues. Fatty acid composition of rat adipose tissue, taken as an indicator of previous dietary fatty acid intake, showed that the DHA content in the adipose tissue of rats receiving the diet supplemented with DHA was >70-fold greater than in the control group ($P < 0.0001$), without any detectable change in n-6 PUFA (Table 2). In a similar way, DHA level was markedly increased in tumor tissues triglycerides (4.6% in the intervention group versus 0.3% in the control group; $P < 0.003$; data not shown). Fatty acid composition of tumor phospholipids was altered with a 2.3-fold increase in DHA level ($P < 0.001$) and a 1.8-fold decrease in n-6 arachidonic acid ($P < 0.001$; Table 2).

α -Tocopherol content of rat tissues. α -Tocopherol was quantified in liver, adipose tissues, tumors, and serum (Fig. 1). In liver (Fig. 1A), α -tocopherol level increased 3-fold in the 100 IU α -tocopherol/kg diet-supplemented group and 6-fold in the 500 IU α -tocopherol group compared with DHA group: median values were 0.403 and 0.920 μ g/mg versus 0.152 μ g/mg lipids, respectively ($P < 0.0002$). In adipose tissue (Fig. 1B), the increase was ~3-fold for the 100 IU group and 4-fold for the 500 IU group: 0.158 and 0.221, respectively, compared with 0.053 μ g/mg lipids for the DHA group ($P < 0.0005$). In tumor tissue (Fig. 1C), the increase was ~2.4-fold for the 100 IU dose and 3-fold for the 500 IU dose: 1.298 and 1.640, respectively, compared with 0.538 μ g/mg lipids for the DHA group ($P < 0.0005$). In serum (Fig. 1D), the increase was ~3-fold for both 100 and 500 IU doses: 1.287 and 1.364, respectively, compared with 0.422 μ g/mg lipids for the DHA group ($P < 0.002$). In all tissue analyses, the level of α -tocopherol was higher in the palm oil group than in the DHA group and was similar to that of DHA plus 100 IU α -tocopherol/kg diet group.

Table 2. Fatty acid composition of rat adipose and tumor tissues

Fatty acids	Adipose tissue*			Tumor tissue*		
	Triglycerides [†]			Phospholipids [†]		
	Control	DHA	Statistics	Control	DHA	Statistics
Saturates						
14:0	0.9 ± 0.1	3.2 ± 0.3	***	0.6 ± 0.2	0.7 ± 0.1	NS
16:0	19.7 ± 1.4	19.3 ± 1.2	NS	24.1 ± 1.8	24.3 ± 0.4	NS
18:0	6.8 ± 1.1	3.2 ± 0.3	***	15.3 ± 1.8	14.2 ± 1.4	NS
Total [†]	28.1 ± 2.0	26.5 ± 1.3	NS	43.4 ± 1.3	42.4 ± 1.4	NS
Monounsaturates						
16:1	2.5 ± 0.6	2.7 ± 0.7	NS	0.9 ± 0.2	1 ± 0.2	NS
18:1n-9c	49.8 ± 1.8	45.4 ± 1.9	**	14.3 ± 1.9	16.9 ± 3	NS
Total [§]	57.0 ± 1.9	52.5 ± 1.7	***	20.8 ± 2.4	23.8 ± 3.3	NS
n-6 PUFA						
18:2n-6c	10.2 ± 1.0	10.2 ± 0.4	NS	2.3 ± 0.4	5 ± 0.9	***
20:3n-6	<0.1	<0.1		0.6 ± 0.06	1.4 ± 0.2	***
20:4n-6	<0.1	<0.1		16.9 ± 1.5	9.2 ± 2.2	***
Total	10.8 ± 1.03	10.5 ± 0.5	NS	21.9 ± 1.9	16.5 ± 2	***
n-3 PUFA						
18:3n-3	0.6 ± 0.08	0.6 ± 0.05	NS	<0.1	<0.1	
22:6n-3	0.09 ± 0.06	6.9 ± 1.2	***	1.8 ± 0.2	4.2 ± 0.9	***
Total [¶]	0.7 ± 0.09	7.5 ± 1.3	***	2.3 ± 0.4	6.6 ± 1.8	***

NOTE: Comparison between control and DHA groups by Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Abbreviation: NS, not significant.

*Sampled from eight randomly selected rats per dietary groups.

[†]Area; %, mean ± SD.

[‡]Including 12:0, 13:0, 15:0, 17:0, 20:0, 21:0, 22:0, and 24:0.

[§]Including 14:1, 15:1, 17:1, 18:1 n-9t, 18:1 n-7c, 20:1, 22:1, and 24:1.

^{||}Including 18:2n-6t, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, and 22:2n-6.

[¶]20:5n-3 nondetectable.

The quantity of α -tocopherol varied proportionately to the lipid content of the tissue analyzed: the lower levels were found in adipose tissues and in the liver; higher quantities were found in tumor tissue and in serum.

Effect of the dietary intervention on tumor response to chemotherapy. Tumor latency was between 7 and 10 weeks after NMU injection according to the nutritional group. At the end of the experimental time, incidence rate was 94.4% in both control and DHA groups. Multiplicity was 3.0 ± 1.5 and 3.7 ± 1.4 tumors per rat (mean ± SD) for control or DHA groups, respectively. The difference between variables was not significantly different between groups, and addition of α -tocopherol did not modify these variables.

Diets had no effect on the rate of tumor growth before beginning of chemotherapy. The effect of diet on the variation of tumor growth, taken as the end point to evaluate tumor response to epirubicin, is presented in Fig. 2A. Tumors regressed in the DHA group by 40%, whereas tumors progressed in the palm group by 30% at the end of the experimental period ($P < 0.01$). This DHA-induced enhancement of efficacy of epirubicin was absent in the group supplemented with 500 IU α -tocopherol/kg diet: tumor size was increased by 10% at the end of the experimental time, and variation in tumor growth in this group was not significantly different from that of the palm oil group. A dose of 100 IU α -tocopherol/kg diet did not abolish the DHA-induced enhancement of tumor response to epirubicin but diminished the response by 25% (not statistically significant).

Effect of the dietary intervention on vascular changes in mammary tumors. PDI mean value was 43% lower in tumors

of rats from the DHA group than in the control, palm group ($P < 0.03$). The difference was observed before initiation of chemotherapy (Fig. 2B). In presence of α -tocopherol (100 or 500 IU), difference in PDI between the palm and the DHA groups was no longer statistically significant, indicating that the effect of DHA on PDI was suppressed by α -tocopherol. Two weeks after the beginning of chemotherapy, the PDI remained decreased by 35% in the DHA group compared with the palm group ($P < 0.03$; data not shown). In the presence of 100 or 500 IU α -tocopherol, the difference with control was no longer significantly decreased (30.8% and 28.8%, respectively). Serum VEGF-A protein level, measured at the time of sacrifice, was higher in the DHA-supplemented group than in the control group (5.0 ± 1.1 and 2.1 ± 0.4 pg/mL, mean ± SE, respectively; $P = 0.01$; Fig. 2C). This increase reached a lower extent in the DHA group receiving low dose of vitamin E (4.2 ± 0.7 pg/mL; $P = 0.02$). In the DHA group with high dose of vitamin E, where tumor response to epirubicin did not differ from control group, VEGF level was not different from control group (3.8 ± 1.0 pg/mL; $P = 0.23$; Fig. 2C). In tumor tissues, VEGF-A level did not differ significantly among dietary groups, from 222 ± 42 in the control group to 338 ± 75 pg/mg proteins in the DHA group. No effect of vitamin E was detectable in tumors: 323 ± 70 and 224 ± 55 in DHA + 100 IU or DHA + 500 IU groups, respectively.

Discussion

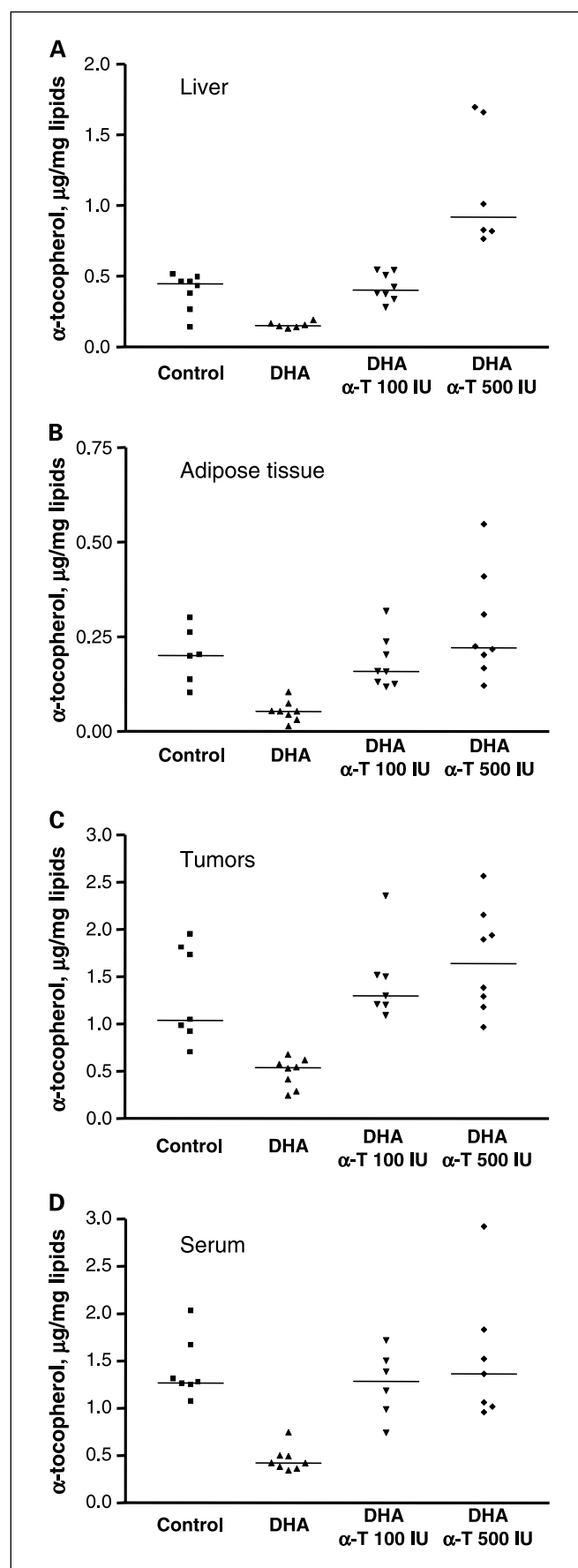
In this study, we have identified a defined nutrient, DHA, which can turn a malignant tumor from resistant to sensitive to

a major anticancer agent in a sound animal model: autochthonous mammary tumors in the female rat. Mammary tumors in the group of rats supplemented with DHA stopped growing after the first injection of epirubicin and tumor size subsequently decreased throughout weekly epirubicin administration. In contrast, mammary tumors in the group of rats on the saturated fatty acid-containing diet kept growing during weekly injections of epirubicin, until they reached a state of stabilization. Thus, dietary supplementation by DHA sensitizes mammary tumors to epirubicin.

We reported previously that patients treated for a breast carcinoma had a better tumor response to neoadjuvant chemotherapy when their adipose tissue contained high level of DHA than when their DHA stores were low (26). Because adipose tissue DHA level reflects past dietary DHA intake (27), this finding suggested that dietary DHA might influence breast tumor sensitivity to chemotherapy. Our experimental demonstration in rats confirms the hypothesis of an increased chemosensitivity under dietary modifications. We have already shown that the membrane fatty acid profile of malignant breast tumors is very similar to that of nontumor breast epithelial tissues (19), indicating that a common factor, such as diet, accounts for this observation. Because lipid membrane composition of tissues relies on the pool of fatty acids, which is mainly modified in organisms by dietary intake, this suggests that chemosensitization of tumors may be explained by changes in their lipid composition, after changes in dietary fatty acids. In our experiment, DHA levels in both adipose and tumor tissue triglycerides were markedly increased in the DHA-supplemented group, indicating that DHA was readily incorporated into both stored and tumor tissue lipids, as well as in cell membranes of tumor, as indicated by the increased DHA level in tumor tissue phospholipids. Indeed, experimental studies indicated that an enrichment of culture medium with DHA increased the sensitivity of a human breast cancer cells to anthracyclines (3). Thus, tumor sensitivity to anticancer agents does not result entirely from the type of genomic alterations present in tumors but is also influenced by dietary lipids.

Peroxidation products resulting from ROS induced by anthracyclines are partly accountable for the enhancement of doxorubicin sensitivity resulting from DHA incorporation into breast cancer cell lines (10). Our study investigated the possibility that the effect of dietary DHA relies on the peroxidability of the fatty acid incorporated into cell membranes and therefore could be abolished by antioxidants, such as α -tocopherol. Such a possibility had already been suggested by results obtained in a previous experiment carried out in rats

Fig. 1. α -Tocopherol level in rat tissues according to dietary intervention. Rats were assigned to four dietary groups: a control group, supplemented with palm oil and three DHA subgroups, which differed by their content in α -tocopherol (α -T), no addition, 100 IU/kg, or 500 IU/kg. Due to differences in tumor growth delay, rats were fed diets for mean lengths extending from 18.5 to 20.9 weeks as described in Materials and Methods. At the time of sacrifice, lipids were extracted from rat liver (A), adipose tissues (B), tumors (C), and serum (D) in each dietary group and α -tocopherol was quantified by reverse-phase high-pressure liquid chromatography as described in Materials and Methods. Symbols, value from one rat tissue; lines, median value. Difference between control and DHA was statistically nonsignificant in (A), significant in (B and C) ($P < 0.05$), and significant in (D) ($P < 0.01$). Differences between DHA and DHA + α -T 100 IU: not significant in (A), $P < 0.05$ in (B and D), and $P < 0.01$ in (C). Differences between DHA and DHA + α -T 500 IU: $P < 0.001$ in (A, B, and C) and $P < 0.01$ in (D). Kruskal-Wallis and then Dunn's multiple comparison test.



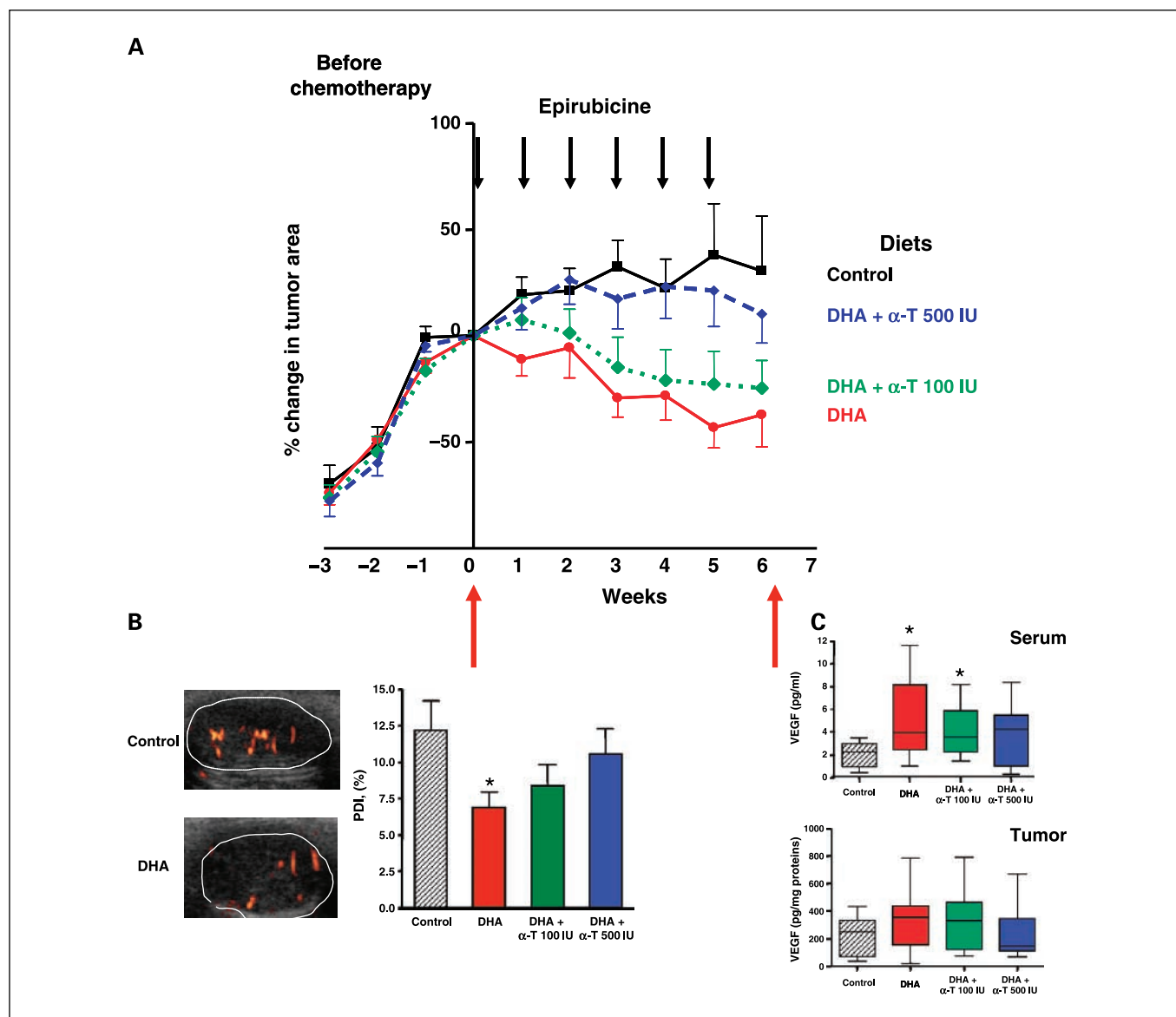


Fig 2. A, tumor size change before and during chemotherapy: rats were assigned to four dietary groups. When the largest tumor reached 1.5 cm², epirubicin was injected once weekly for 6 weeks (arrows, injections). The beginning of chemotherapy was set as the reference for determining the percentage variation in tumor size according to time. Points, mean of tumor size change in each group (n = 16); bars, SE. After beginning of chemotherapy, difference between control and DHA conditions: P < 0.01; between DHA and DHA + α -T 500 IU conditions: P < 0.05. Repeated measured ANOVA with grouping factor (time). B, functional imaging (left) and quantification (right) of tumor vascularization before beginning of chemotherapy. Tumor was individualized by sonography (dashed circles) and blood flow was analyzed by power Doppler and quantified by color pixel detection using a dedicated software. PDI was calculated in each tumor using the mean value of five stored images of a tumor to reflect the mean PDI of the tumor. Representative detection of vessel is shown in a rat from the control or DHA groups. Level of tumor vascularization (PDI; %) for each dietary condition (right). Columns, mean (n = 14); bars, SE. *, P < 0.03 for control and DHA conditions. C, serum and tumor VEGF-A protein level. Level of VEGF-A was measured at the time of sacrifice in the four dietary groups. Lines within boxes, median values (n = 9); bars, SD. *, P < 0.05 for control and DHA and for control and DHA + α -T 100 IU.

receiving fish oil (11). Using DHA in the present study, we found that α -tocopherol produced a dose-dependent suppression of this effect with no difference in tumor sensitivity between the group of rats receiving the highest amount of α -tocopherol and the control group receiving saturated fatty acids. Thus, chemosensitization by DHA is strongly influenced by the level of antioxidant provided. The results of α -tocopherol quantification in all tissues analyzed (liver, adipose tissues, serum, and tumors) showed that enrichment in this compound is dependent on the dose of α -tocopherol added to the food. However, the greatest difference between α -tocopherol groups was observed in the liver, an expected

finding because the liver stores this compound (28). Level of α -tocopherol in all tissues analyzed was greater in the palm oil group than in the DHA group and was similar to that of rats receiving 100 IU α -tocopherol/kg. This difference in α -tocopherol level between tissues of the DHA or palm oil groups was not found in the corresponding diets, suggesting that it was the consequence of a greater consumption of α -tocopherol in the rat tissues of the DHA group than in the palm oil group. This may be explained by a greater need for defense against peroxidation when DHA was given to the rats. We do not know if the tumor response to DHA is secondary to the formation of an epoxide within the fatty acid, which brings

about changes in other cellular molecules. For instance, the growth-inhibitory effects of n-3 fatty acids in some tumor cells has been related to changes in intracellular calcium, which have also been reported to be modified by vitamin E (29).

The mechanisms accounting for tumor chemosensitization by DHA may involve epithelial tumor cell response to anthracyclines or host factors, such as tumor vascularization. Because tumor chemosensitivity is influenced by local vascularization, we also investigated whether dietary DHA might modify tumor tissue blood flow. For the quantification of tumor vascularization, we used power Doppler sonography as a method developed *in vivo* in small animals. Power Doppler sonography was used for the first time in human breast tumor studies 20 years ago and has been validated in providing overall assessment of tumor vascularization (30). This noninvasive technique offers many advantages, such as high sensitivity, simplicity of use, and repeatability (31). The recent development of high-frequency probes makes power Doppler sonography suitable for use in our rat model of chemically induced mammary tumors and provides a high reproducibility of vascular quantification *in vivo* (21). Using this approach to evaluate tumor vascularization, we found that tumor vascularization was lower in the group of rats supplemented with DHA than in the control group, even before anthracyclines treatment was initiated. The fact that inhibition tends to disappear when α -tocopherol is added suggests that tumor vascularization might contribute to the chemosensitization induced by DHA. This tumor vascularization remained lower in the DHA group throughout epirubicin treatment.

There is an apparent contradiction between the decrease in tumor vascularization induced by DHA and the enhancement of tumor sensitivity to anthracyclins because this effect is expected to rely on the production of ROS, which depends on tumor oxygenation. It could be speculated that decreased tumor perfusion induced by the DHA diet leads to an increase in hypoxic areas. Because ROS are specifically generated in the mitochondria during hypoxia by disruption of the oxidative phosphorylation (32), the increase in tumor sensitivity induced by DHA could result from decreased tumor oxygenation. This hypothesis fits with the observation that α -tocopherol, which suppresses ROS production, also suppresses DHA-mediated tumor growth inhibition.

It should be stressed that Doppler sonography investigates a part of tumor vascularization because the technique is sensitive only to large vessel flow (i.e., arterioles and venules larger than $\sim 100 \mu\text{m}$). We are currently considering the need to develop new approaches to visualize tumor microcirculation and determine whether dietary DHA has an effect on it, similar to that observed on macrovascularization.

The potential antiangiogenic effect of DHA is in line with results of *in vitro* studies, which found an inhibitory effect of n-3 PUFA on proliferation and/or on tube-forming activity of bovine aortic endothelial cells, suggesting a potential inhibitory effect of n-3 PUFA on angiogenesis (33, 34). Moreover, *in vivo*

studies showed that n-3 PUFA-induced inhibition of tumor growth is accompanied by a decreased angiogenesis, evaluated by immunohistochemistry in a model of implanted MDA-MB-231 human breast cancer cell line in athymic nude mice (35) or by measure of VEGF expression in a rat model of implanted fibrosarcoma cells (36) or in human colon cancer cells (37). In our rat system of mammary tumors, VEGF protein level was not decreased in tumor tissues of groups receiving DHA. There was even an increased VEGF level in serum of the DHA group compared with controls and a trend for an increase in tumors. This serum VEGF increase was observed after the end of chemotherapy and may result from an increased VEGF response of the tumor to tumor ischemia or necrosis in the DHA group compared with controls because tumor vascularization was decreased in the DHA group before and during chemotherapy. In line with this hypothesis, the lack of significant increase in serum VEGF in the high-tocopherol group compared with controls was associated with a lack of decrease in tumor vascularization. Thus, vascularization seems to have a role in the DHA-induced sensitization of tumors to chemotherapy.

We do not know presently whether the changes in tumor sensitivity to anthracyclines observed in rats after a dietary supplementation could be transposed to humans. We actually used very high quantity of DHA in rats, which may amount for about 20- to 50-fold the mean estimated DHA intake in humans. In a recent phase I to II clinical trial carried out in metastatic breast cancer patients, we found that a daily supplementation of 1.8 g DHA during chemotherapy led to a doubling of the serum phospholipid DHA level within 10 days (38). In human, the mean DHA level within phospholipids of breast carcinoma has been shown to vary between undetectable to 3.5% of total fatty acids (19). Because the main determinant of variation is diet, it can be assumed that DHA level in tumors would be influenced by supplemental dietary DHA.

In conclusion, our study indicates that DHA enhances tumor response to chemotherapy. This effect, suppressed by α -tocopherol, is dependent on the amount of α -tocopherol added to the food. It is also associated with a decrease in tumor vascularization, which occurs before the beginning of chemotherapy and is sustained during the treatment time period. Our results may help to understand the possible mechanisms involved in the effects of dietary components on tumor sensitivity to anticancer agents. The ability of vitamin E to counter the antitumor effects of the DHA-epirubicin association has to be taken into account, given the widespread consumption of this supplement. These results may potentially lead to use of ω -3 PUFA as dietary adjuvants to anticancer treatments in patients, although appropriate clinical trials should be carried out before directly transposing to humans the data obtained in rats.

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