α-Tocopherol Suppresses Mammary Tumor Sensitivity to Anthracyclines in Fish Oil–Fed Rats

Séverine Colas, Emmanuelle Germain, Khelifa Arab, Karine Maheo, Caroline Goupille, and Philippe Bougnoux

Abstract: Polyunsaturated fatty acids (PUFAs) have been reported to enhance the efficacy of chemotherapeutic agents that produce reactive oxygen species such as anthracyclines. We previously reported in a human breast cancer cell line that the increased cytotoxic activity of anthracyclines by several PUFAs was abolished by antioxidants and enhanced by pro-oxidants, suggesting that lipid peroxidation was involved in this effect. To determine the relevance of this observation in vivo, we examined the effect of the oxidative status of the diet on the activity of epirubicin against N-methyl-nitrosourea–induced mammary tumors in Sprague-Dawley rats. Three groups of rats were fed a basal diet enriched with dietary n-3 PUFA (sardine oil, 15%) alone (control group), with addition of an antioxidant (α-tocopherol, 100 UI/kg diet), or with addition of an oxidant system (dehydro-ascorbate/naphthoquinone). When the first mammary tumor reached 1 cm², epirubicin was administrated weekly for 3 wk, and subsequent change in tumor size was documented over time. Two weeks after the end of epirubicin injections, tumor size was increased by 34% in the control group. In the pro-oxidant group, tumor size was decreased by 50%. In contrast, tumor size was increased by 188% in the antioxidant group. Thus, addition of pro-oxidants in a fish oil–enriched diet increased the sensitization of mammary tumors to chemotherapy, whereas addition of α-tocopherol suppressed tumor response in vivo, indicating that interaction between components of the diet has to be carefully controlled during chemotherapy.

Introduction

Chemotherapy represents a major treatment for various stages of cancer. Increasing tumor tissue sensitivity to anticancer drugs remains a challenging issue to improve treatments. Recent advances have documented the fact that dietary components may influence the efficacy of several anticancer agents. Dietary lipids, in particular, n-3 polyunsaturated fatty acid (PUFA), have already been suggested to modify the sensitivity of mammary tumors to reactive oxygen species (ROS)-generating anticancer drugs (1). Among PUFAs, eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) have particular potential to enhance cytotoxicity of various anticancer drugs (2–4). Experimental evidence has shown that n-3 PUFA might enhance tumor response to drugs in vitro (5–7) and in vivo (8–11). The possibility that a similar mechanism operates in humans was explored by using adipose tissue as a biomarker of the n-3 PUFA status in patients receiving neo-adjuvant chemotherapy for an initially localized breast carcinoma. Level of DHA in adipose tissue was higher in the group of patients with complete or partial response of the breast tumor to chemotherapy than in patients with no response or with tumor progression (12).

Several mechanisms have been involved in the enhancement of tumor response to cytotoxic drugs. Among hypotheses, enhancement of tumor cell membrane lipid peroxidation induced by anticancer drugs may be responsible, at least in part, for the effects of PUFA. These effects have been found to be proportional to the degree of unsaturation (13). Moreover, the increased cytotoxicity of doxorubicin in the presence of DHA was associated with an increased generation of lipid hydroperoxides (5), an observation already reported in another cell system with gamma linolenic acid (GLA) (14). Furthermore, the increasing effect of DHA on doxorubicin cytotoxicity has been shown, in vitro, to be enhanced by the addition of an oxidant system or to be abolished by the addition of the antioxidant α-tocopherol (5). In vivo, enhancement of radiosensitivity of mammary tumors by dietary DHA has been found to be inhibited by the addition of α-tocopherol (15).

All these observations suggest that dietary enrichment of tissues in peroxidizable lipids such as DHA may bring an additional target to ROS-generating anticancer drugs, resulting in enhanced sensitivity of tumors to these agents, provided there is no antioxidant to abolish this effect. To investigate this hypothesis, we used a model of autochthonous rat mammary tumors and carried out a dietary intervention with fish...
oil rich in n-3 PUFA and examined whether tumor response to epirubicin, a major anticancer agent used in breast cancer chemotherapy, was modified by the oxidative status of the diet.

Materials and Methods

Animals and Experimental Carcinogenesis

Thirty-six female Sprague-Dawley rats were purchased from IFFA CREDO (L’Arbresle, France) when they were 43 days of age. The care of these animals was in accordance with institution guidelines. They were housed three per cage and maintained in a temperature- and humidity-controlled environment (22°C) with a 12-h light/dark cycle. To initiate mammary tumors, rats received a single dose of N-methylnitrosourea (NMU, Sigma, Saint Quentin Fallavier, France) at 48 days of age by a subcutaneous injection (25 mg/kg body weight) as previously described (16).

Diets

At the time of their arrival, all rats received a basal diet that included casein (22 g/100 g), methionine (0.16 g/100 g), corn starch (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, B3-9, B12, and C (1 g/100 g) (obtained from INRA, Jouy en Josas, France). This basal diet was supplemented with 6% sardine oil (Polaris Biotechnique, Concarneau, France), which is in accordance with lipid supply in rat (17).

At the time NMU was administered, animals were randomly divided into 3 groups of 12 animals each. All rats received the same basal diet than before NMU administration but enriched with 15% sardine oil. The control group received no additional supplementation, the antioxidant group was supplemented with vitamin E (α-tocopherol acetate, 100 UI/kg diet), and the pro-oxidant group was supplemented with the redox system dehydroascorbate/naphthoquinone (20 and 0.2 mg/day, respectively, diluted in drinking water) as previously described by Noto et al. (18). Animals were fed ad libitum and had similar food intake. Experimental diets were prepared weekly and kept at 4°C until used. The oxidant mixture was prepared just before addition to drinking water. Food and water were replaced every day.

Assessment of Tumor Chemosensitivity

Four weeks after NMU induction, rats were weighed and palpated twice a week to determine the location, latency, incidence, and multiplicity of NMU-induced tumors. Tumor size was measured with a caliper, and tumor area was calculated as the product of the two longest diameters. As soon as the first mammary tumor (target tumor) reached 1 cm², epirubicin (freshly prepared in saline) was injected by intraperitoneal route at a dose of 3 mg/kg once a week for 3 wk to reach 9 mg/kg as the cumulative dose. To evaluate the chemosensitivity of tumors to epirubicin, results were expressed as percent of change of the tumor surface over time. Tumors were assessed during all the chemotherapy time period and for two additional weeks.

Tissue Lipids Analysis

At the end of the experiment, animals were humanely sacrificed and necropsied. Adipose and tumor tissues were harvested, rinsed in saline, and immediately frozen and kept in liquid nitrogen for biochemical analysis. Eight rats were randomly selected in each dietary group to provide adipose tissues for fatty acid determination. Determination of the fatty acid content of the adipose tissue was performed as previously described (19). After lipids were extracted according to the Folch extraction procedure (20), triglycerides of the adipose tissue were purified by preparative thin layer chromatography and fatty acids were methylated with boron trifluoride and analyzed by capillary gas chromatography using a 50-m polar column and an on-column injector as described (21). Fatty acids were expressed as a percentage of total peak area.

Tumor α-Tocopherol Analysis

Rats were randomly selected in each dietary group to provide tumor tissues for α-tocopherol determination. Total lipids were extracted from tumors according to the Bligh and Dyer extraction procedure (22). α-Tocopherol was directly extracted with hexane without prior saponification (23). Samples from rats were analyzed by high-pressure liquid chromatography (HPLC) (23). This system consists of an 80-μl injection loop; the solvent was methanol and was pumped at 1 ml/min through a 250 × 4.6-mm reverse-phase octadecyl-bonded silica column (Waters Spherosorb ODS2, ThermoFinnigan, Les Ulis, France). Detection of tocopherols was performed with a fluorometer (FL2000, ThermoFinnigan) with excitation wavelength of 290 nm and detection at 334 nm coupled with an SP4270 Integrator (Spectraphysics, ThermoFinnigan). Separated compounds were quantified by comparison of their peak areas with that of δ-tocopherol used as an internal standard.

Malondialdehyde (MDA) Status of Tumors

Immediately after sacrifice, tumor tissues were stored in liquid nitrogen. Before analysis, one piece of 50 mg was thawed and homogenized in 400 μl of Tris-HCl 100 mM buffer with ethylene diaminetetra acetic acid (EDTA, 1 mM), butylated hydroxytoluene (BHT, 0.1 mM), and phenylmethanesulfonyl fluoride (PMSF, 0.1 mM). The tissue was homogenized with a small quantity of sand, the extract was centrifuged at 2,000 g for 4 min, and the supernatant was used to measure total MDA and proteins. MDA was derivatized with methylmethanesulfonamide in an acidic medium at 37°C and measured as a diazepinum derivative by HPLC-UV (24) with mass spectrometric confirmation. Proteins were measured
with bicinchoninic acid (BCA) assay protein quantification kit from Pierce (Montluçon, France) with a BSA standard.

Statistical Analysis

Results on tumor size regression were expressed as mean ± SE. Repeated-measures analysis of variance with grouping factor (time) was used to compare dietary groups as a function of diet and time after normality of distribution was determined. To determine whether differences in the mean values for fatty acid analyses were significant, a Mann-Whitney test was used. To determine whether differences in the mean values for α-tocopherol or MDA analyses in all groups were significant, a Kruskal-Wallis test was used. Then, to highlight differences between two groups, Dunn’s test was used. The level of statistical significance was set at \( P < 0.05 \).

Results

Fatty Acids Analysis

Fatty acids composition of sardine oil is reported in Table 1. DHA and EPA levels reach, respectively, 12% and 18.5% of total fatty acids. To ensure that n-3 PUFAs supplied through fish oil–enriched diet were actually integrated into body tissues, we measured the fatty acid adipose tissue composition as a reflection of body stores. Fatty acid pattern of adipose tissue is shown in Table 1. Main PUFAs were DHA and EPA, which accounted for most of n-3 PUFAs. Respective proportions of EPA, 22:5n-3 [docosapentaenoic acid (DPA)], and DHA in adipose tissue were distinct from that found in sardine oil. Although EPA was preponderant in sardine oil, DHA was the major n-3 PUFA in adipose tissue. The addition of α-tocopherol or of the pro-oxidant system did not alter adipose tissue fatty acid pattern.

Tumor Response to Chemotherapy

During the experiment, no significant differences in body weight among dietary groups were observed (data not shown). Not all the rats developed tumors during the experimental time (mean incidence was about 94%). The size of the largest tumor was sufficient to initiate chemotherapy in 10 of 12 rats in the control group, 9 of 12 rats in the pro-oxidant group, and 9 of 12 rats in the antioxidant group. The average time for tumors to reach 1 cm² was about 11 wk after NMU injection with no differences between groups.

The effect of dietary intervention on tumor size variation according to the chemotherapy time and for 2 wk after the end of treatment is shown in Fig. 1. There were significant differences in tumor response to chemotherapy among the three nutritional groups (\( P < 0.0001 \)). We observed a stabilization of tumor growth in control rats (+34% size increase, 4 wk after the first injection of epirubicin). In contrast, tumor size of rats receiving the additional pro-oxidant mixture was decreased by 50% (\( P < 0.03 \)). In the antioxidant group (α-tocopherol) tumor size increased by 188% (\( P < 0.03 \)).

Table 1. Fatty Acid Composition of Fish Oil Diet and Rat Adipose Tissue

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Sardine Oil (%)</th>
<th>Adipose Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Saturates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>7.2</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>15</td>
<td>24.6 ± 0.9</td>
</tr>
<tr>
<td>18:0</td>
<td>3.1</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>Total</td>
<td>25.1</td>
<td>37.2 ± 1.7b</td>
</tr>
<tr>
<td>Monounsaturates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>8.3</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>18:1n-9c</td>
<td>13</td>
<td>30.3 ± 0.9</td>
</tr>
<tr>
<td>20:1</td>
<td>&lt;1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Total</td>
<td>21.3</td>
<td>42.6 ± 1.3c</td>
</tr>
<tr>
<td>n-6 PUFA</td>
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<td></td>
</tr>
<tr>
<td>18:2n-6c</td>
<td>1.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>&lt;1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>1.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>22:2n-6</td>
<td>&lt;1</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Total</td>
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<td>5.3 ± 0.3d</td>
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<td></td>
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<tr>
<td>18:3n-3</td>
<td>2</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>18.5</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>2.5</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>12</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>12.2 ± 0.6</td>
</tr>
</tbody>
</table>

\( a \): Values are mean % area ± SD sampled from eight randomly selected rats per dietary group. PUFA, polyunsaturated fatty acid.

\( b \): Including 12:0, 13:0, 15:0, 17:0, 20:0, 22:0, and 24:0.

\( c \): Including 14:1, 15:1, 17:1, and 22:1.

\( d \): Including 18:2 n-6, 20:2 n-6, and 20:3n-6.
Redox Status of Mammary Tumors

α-Tocopherol level (Fig. 2A) was increased by 32-fold in tumors of the α-tocopherol–supplemented group compared with the control or pro-oxidant groups (P < 0.002). MDA level (Fig. 2B) was increased in the pro-oxidant group compared with the control group: 474 vs. 187 nmol/mg protein, median values, respectively, but the difference was not statistically significant due to a wide dispersion of measures. In contrast, MDA level was not influenced by addition of α-tocopherol compared with the control group.

Discussion

Several animal studies have demonstrated that the consumption of ω-3 fatty acids can increase the efficacy of chemotherapy (8,9,25–27) or radiotherapy (15). Mechanisms that might account for this beneficial effect of n-3 PUFA are not presently known. Hypotheses include enhancement of cancer cell apoptosis, decreased angiogenesis or cell proliferation, altered regulation of cytokine-dependent cell growth or eicosanoid production, and increased uptake of anticancer agents (28). Lipid peroxidation secondary to PUFA enrichment of cell membranes may represent an additional mechanism (14).

Our study shows that dietary components other than fatty acids influence the efficacy of epirubicin on mammary tumors enriched in n-3 PUFA. When rats were fed a diet enriched with fish oil, addition of a pro-oxidant mixture enhanced the effect of chemotherapy. In contrast, addition of α-tocopherol decreased the effect of chemotherapy. Biochemical analyses demonstrate that diets had an impact on tissue composition. First, analysis of fatty acids showed that rat adipose tissues were enriched in DHA and EPA by the dietary supply of fish oil to reach 6.7% and 2.8%, respectively. This fatty acid profile reflects the efficiency of the dietary intervention to alter rat tissue composition because we know that adipose tissues of rats without marine oil supplementation contain a very low level of n-3 PUFA and that fatty acid composition in mammary tumors was affected by the dietary supplementation in a way similar to that of adipose tissues (15). Second, the α-tocopherol supply in the diet actually influenced the α-tocopherol content of tumors because tumor level of this compound was increased by 32-fold in the α-tocopherol dietary group compared with the control group. Contrary to what we had expected, MDA, a highly reactive three-carbon dialdehyde bioproduct of PUFA peroxidation, was not significantly increased in the pro-oxidant group and
was not influenced by the addition of α-tocopherol. Thus, our data failed to unambiguously document lipoperoxidation as the mechanism by which n-3 PUFAs increase epirubicin efficacy and could indicate that mechanisms other than lipid peroxidation may be operating in the effect of fish oil.

Several studies have reported that PUFA may inhibit cell proliferation by mechanisms additional to lipoperoxidation. In cell culture, addition to the medium of α-tocopherol did not totally reverse PUFA-induced cell proliferation inhibition (29,30). There are conditions where lipoperoxidation may even not be involved; the cytotoxicity of paclitaxel, a drug that has not been shown to induce oxidative stress, was reported to be enhanced by other PUFAs such as GLA in breast cancer cells (31). In our experiment, the pro-oxidant system used did not lead to an increased level of MDA in tumors, whereas the cytotoxic effect of the anthracycline (epirubicin) was enhanced, suggesting that an action of ROS independent of oxidative stress could account for this effect. This pro-oxidant system may have generated ROS in amounts insufficient to induce lipid peroxidation but sufficient enough to act as signaling molecules. Actually, several studies have demonstrated that superoxide anion and hydrogen peroxide can behave as signal molecules. For example, diverse proapoptotic stimuli including superoxide anion and chemotherapeutic drugs have been shown to cause mitochondrial depolarization and cytochrome c release, a critical event provoking a cascade of caspase activation and triggering apoptosis (32,33). The signaling function of ROS has also been involved in the activation of T lymphocytes through their specific antigen receptor (TCR) (34), as in the activation of tyrosine-kinase receptor specific to growth factors, in the activation or inactivation of transcription factors or in lipid signaling and in numerous other signaling pathways (35). Finally, vitamin K3 by itself may act on anthracycline activity in cancer cells through mechanisms independent of oxidation (36,37).

In our study, the inhibition of tumor response to epirubicin observed when α-tocopherol was added may also result from mechanisms other than its antioxidant effects. For instance, α-tocopherol has been reported to regulate the expression of several genes implicated in various cellular signaling pathways (38,39). In addition, there is increasing evidence that the isoforms of vitamin E are involved in distinct signaling pathways (40–42). This point may be relevant to the apparently contradictory effect between our results and those reported by Liu and Tan, who found that vitamin E along with n-3 PUFA might improve the outcome after chemotherapy (43). Liu and Tan used a mixture of tocopherols with γ-tocopherol being in fourfold excess over α-tocopherol, whereas we used strictly α-tocopherol to alter the redox status in favor of the antioxidants. In addition, their study did not address the same aspects of drug efficacy because they examined the effect of their dietary intervention on survival of mice grafted with lymphoma cells, whereas we used as an end-point tumor regression after chemotherapy in rats. In our study, it should be stressed that treatments were applied to fully developed mammary tumors to reproduce the situation found in clinical oncology where breast tumors are treated while they have reached advanced stages of tumor development.

In conclusion, our results indicate that several dietary components interact with n-3 PUFA to influence the response of mammary tumors to an anthracycline. This result is of importance because several studies suggest that n-3 PUFA, in particular, DHA, could be used as a dietary adjuvant to radio- or chemotherapy in patients treated for cancer. Our experiments show that the interaction between components of diets must be well controlled in such a new paradigm of cancer treatment. This emphasizes the growing need to understand the molecular mechanisms involved in the effects of dietary components on tumor sensitivity to anticancer agents.

Acknowledgments and Notes

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