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ENHANCEMENT OF DOXORUBICIN CYTOTOXICITY BY POLYUNSATURATED FATTY ACIDS IN THE HUMAN BREAST TUMOR CELL LINE MDA-MB-231: RELATIONSHIP TO LIPID PEROXIDATION

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Exogenous polyunsaturated fatty acids modulate the cytotoxic activity of anti-cancer drugs. In this study, we examined whether lipid peroxidation is a potential mechanism through which fatty acids enhance drug cytotoxicity. We measured cell viability in the human breast cancer cell line MDA-MB-231 exposed to doxorubicin in the presence of non-cytotoxic concentrations of various polyunsaturated fatty acids for 6 days. To determine the role of lipid peroxidation, the hydroperoxide level was measured in cell extracts. Among all polyunsaturated fatty acids tested, docosahexaenoic acid (DHA, 22:6n-3) was the most potent in increasing doxorubicin cytotoxicity: cell viability decreased from 54% in the presence of 10^{-7} M doxorubicin alone to 21% when cells were incubated with doxorubicin and DHA. After addition of an oxidant system (sodium ascorbate/2-methyl-1,4-naphthoquinone) to cells incubated with doxorubicin and DHA, cell viability further decreased to 12%. Cell hydroperoxides increased commensurately. The effect of DHA on doxorubicin activity and lipid hydroperoxide formation was abolished by a lipid peroxidation inhibitor (dl-α-tocopherol) or when oleic acid (a non-peroxidizable fatty acid) was used in place of DHA. No effect was observed with mitoxantrone, a drug with a low peroxidation-generating potential. Thus, DHA may increase the efficacy of oxyradical-producing drugs through a mechanism involving a generation of lipoperoxides. This may lead *in vivo* to a modulation of tumor cell chemosensitivity by DHA and oxidant agents. *Int. J. Cancer* 75:578–583, 1998.
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Several experimental studies have shown that exogenous polyunsaturated fatty acids (PUFAs) may sensitize tumor cells to anti-cancer drugs in cell culture (Burns and North, 1986; Zijlstra *et al.,* 1987; Petersen *et al.,* 1992) or in tumors growing in animals (Shao *et al.,* 1995). No precise mechanisms of action of PUFA in the modulation of anti-cancer drug efficacy have been been determined. It has been proposed that the enhanced killing effect of anti-cancer drugs could result from alterations in the biophysical properties and functions of membranes brought about by PUFA supplementation. Indeed, supplementation of growth medium with docosahexaenoic acid (DHA, 22:6n-3) was found to sensitize resistant human small-cell lung cancer cell lines to doxorubicin by increasing intracellular drug concentrations (Zijlstra *et al.,* 1987). In a similar way, the increase of the fatty acid unsaturation level in the membrane of the sensitive L1210 cell line was correlated with the increase of cellular accumulation of cytotoxic drugs (Burns and North, 1986).

In addition to PUFA effects on membrane structure, peroxidation of highly unsaturated fatty acids was linked to cytotoxicity in neoplastic cells (Bégin *et al.,* 1988). In response to oxidative stress, PUFAs undergo free-radical chain reaction breakdown, which results in the formation of fatty acid hydroperoxides and hydroxides as immediate products and aldehydes, among other end-products. Either in cultured tumor cells (Chajès *et al.,* 1996) or in tumor-bearing animals (Gonzalez *et al.,* 1993), PUFA supplementation caused a decrease in cell proliferation or in tumor growth correlated with a concomitant increase in the level of cellular lipid peroxidation. These observations suggest that PUFAs interfere with tumor cell proliferation *in vitro* and *in vivo* due to the formation of oxidation products. A number of lipid hydroperoxides and lipid peroxidation-derived aldehydes have been implicated as causative agents for cytotoxic processes in neoplastic cells. Aldehydes supplemented to tumor cell culture (Hauptlorenz *et al.,* 1985) or injected in tumor-bearing animals (Tessitore *et al.,* 1989) decrease the rate of tumor cell proliferation.

Little is known about the involvement of lipid peroxidation in the modulation of drug efficacy by PUFAs. The metabolism of some quinone-containing anti-cancer drugs, such as doxorubicin and mitomycin C, yields oxygen-reactive species. These in turn could react with the PUFA double bonds and induce the lipid peroxidation process (Benchekroun and Robert, 1992). In a doxorubicin-resistant mammary tumor cell line, lipid peroxidation level was found to be lower than in the wild-type cell line, suggesting a relationship between lipid peroxidation and sensitivity of tumor cells to doxorubicin (Benchekroun *et al.,* 1993). The increased membrane unsaturation index consequently would provide more abundant targets for peroxidation events generated by the metabolism of doxorubicin or that of mitomycin C and, thus, increase drug efficacy. In agreement with this hypothesis, a correlation between doxorubicin sensitivity and the degree of polyunsaturation in membranes has been reported in L1210 cells (Burns and North, 1986). Similarly, in tumor-bearing animals, a highly unsaturated fat diet compared to a control low-fat diet exacerbated the responsiveness of MX-1 human mammary carcinoma to mitomycin C by increasing the susceptibility of tumor cells to mitomycin C-induced oxidative stress (Shao *et al.,* 1995).

In this study, we investigated whether exogenous PUFAs could enhance the cytotoxic activity of anti-cancer drugs which generate oxidative stress through an increase in the susceptibility of tumor cells to lipid peroxidation. We quantified the ability of different PUFAs to modulate the sensitivity of the human breast tumor cell line MDA-MB-231 to anti-cancer drugs and examined whether inducing or inhibiting lipid peroxidation altered this effect. Doxorubicin was chosen for its ability to generate reactive oxygen species, and mitoxantrone, usually considered to be unable to induce *in vitro* peroxidation events, was tested as a control drug. In addition, the relationship between cellular lipid peroxidation products and drug toxicity was investigated.

MATERIAL AND METHODS

Mitoxantrone was purchased from Lederle (France) and doxorubicin from Dakota (France). Stock solutions stored at −20°C (9×10^{-4} M for doxorubicin and 4×10^{-3} M for mitoxantrone) and dilutions of doxorubicin and mitoxantrone were freshly prepared in physiological saline solution, NaCl 0.9%.

DHA (22:6n-3), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), γ-linolenic acid (18:3n-6), α-linolenic acid (18:3n-

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3), linoleic acid (18:2n-6) and oleic acid (OA, 18:1n-9) (≥99% purity) were purchased from Sigma (St. Louis, MO) and used as methyl esters, as already described (Goré *et al.,* 1994). The fatty acids were dissolved in 99% ethanol and stored as stock solutions (10 mg/ml) under nitrogen at −20°C. For all experiments, fatty acids were prepared freshly from stock solutions and diluted with growth medium. Vitamin E (dl-α-tocopherol), sodium ascorbate and 2-methyl-1,4-naphthoquinone were obtained from Sigma. The color reagent of the commercially available M-CHO kit for the enzymatic determination of cholesterol was purchased from Merck (Darmstadt, Germany).

Cell culture

The human breast carcinoma cell line MDA-MB-231 was cultured in DMEM containing 5% FCS, 1% penicillin/streptomycin. Cells were seeded at 1×10^4 cells per well in 24-well tissue culture plates (cell viability experiments) or at 3×10^5 cells per well in 6-well tissue culture plates (lipid hydroperoxide determination). Cells were grown in growth medium at 37°C in a 95% air, 5% CO_2-humidified incubator. One day after seeding, the medium was removed and fresh medium along with various concentrations of cytotoxic drugs, in the presence or absence of fatty acids, oxidant, anti-oxidant or combinations of compounds, were added to cultures in parallel. Combined treatments were made using fatty acids, oxidant mixture and anti-oxidant as specified. Solutions of each cytotoxic drug were freshly diluted with culture medium to give specified final concentrations. Solutions of each fatty acid in ethanol were diluted with medium to give a final concentration of 34 μM for oleic, linoleic, α-linolenic and γ-linolenic acid; 32 μM for arachidonic and eicosapentaenoic acid; and 29 μM for DHA. Oxidant mixture was made of a combination of sodium ascorbate at a final concentration of 10^{-4} M and 2-methyl-1,4-naphthoquinone at 10^{-6} M, according to Noto *et al.* (1989). Anti-oxidant vitamin E in ethanol was diluted with culture medium to give a final concentration of 10 μM. The final ethanol concentration in the medium was below 0.15% (v/v). Control cultures received fresh medium containing 0.15% ethanol and were incubated in parallel. The medium was changed every 48 hr until day 6.

Cell viability

To evaluate drug activity, we chose to quantify cell viability; 1×10^4 cells in 500 μl of growth medium were seeded in each well (24-well trays) and incubated as described above for up to 6 days. Every 48 hr, the medium was replaced with fresh identical medium and detached cells were collected in each well and kept at 4°C. At indicated times, adherent cells were washed 3 times with PBS and detached with 200 μl of trypsin (500 mg/l)/EDTA (200 mg/l). After 5 min at 37°C, the reaction was stopped with the respective medium containing the detached cells previously pooled. Then, the cell suspension was incubated with Trypan blue/PBS (1:1) for 10 additional min. For each well, cell viability was evaluated as the number of viable cells × 100/total number of cells. Cell counts were performed for all cultures from 3 experiments in which triplicate measurements were made. Results were expressed as mean values ± SE.

Iodometric assay for the measurement of lipid hydroperoxides

For the peroxide assay, we used the color reagent of the commercially available kit for the enzymatic determination of cholesterol. The content of lipid hydroperoxides in the total lipid extract of tumor cells was determined as previously described for the determination of lipid hydroperoxides in human serum samples (El Saadani *et al.,* 1989) and in breast tumor cells in culture (Chajès *et al.,* 1996). This assay is based on the conversion of iodide to tri-iodide due to the oxidative capacity of lipid hydroperoxides. Tri-iodide can be measured photometrically at 365 nm. MDA-MB-231 breast tumor cells were seeded at 3×10^5 cells in 2.5 ml of growth medium per well (6-well trays). After 24 hr at 37°C, cell cultures were supplemented with cytotoxic drugs in the presence or absence of fatty acids, oxidants or combinations of fatty acids, oxidants and anti-oxidant. Control cultures received fresh medium with 0.15% ethanol and were incubated in parallel. One day after supplementation, cells were trypsinized, harvested and washed 3 times with PBS. After keeping an aliquot for determination of the protein content according to Lowry *et al.* (1951), total lipid extraction was performed with chloroform/methanol (Bligh and Dyer, 1959). The chloroform phase was evaporated under a stream of nitrogen and the dry residue solubilized in 50 μl of ethanol. Samples were mixed on a vortex mixer with 1.0 ml of the color reagent and incubated for 30 min in the dark at room temperature. Absorbance was measured at 365 nm against the color reagent as blank. Lipid hydroperoxide measurements were performed in duplicate in 3 independent experiments. Results were expressed as mean ± SE. Cumen hydroperoxide was used as a standard and a linear relationship was observed between the amount of hydroperoxide and the concentration of tri-iodide produced (slope = 0.92).

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney test. Differences were considered significant at $p \leq 0.05$.

RESULTS

Effect of DHA on doxorubicin-induced cell toxicity

To determine the cytotoxic activity of doxorubicin, a dose effect experiment was performed. After 6 days of culture, 10^{-7} M doxorubicin caused cell viability to drop to 54 ± 2%. Therefore, all subsequent experiments were carried out at 10^{-7} M doxorubicin.

To evaluate the cytotoxic effect of the highly unsaturated (DHA, 22:6n-3) and low unsaturated (OA, 18:1n-9) fatty acids on MBA-MB-231 cells, a dose effect curve was performed. DHA at 29 μM and OA at 34 μM had no effect on tumor cell viability during 6 days of culture (Fig. 1). Hence, all of the following experiments were conducted with 29 μM for DHA and 34 μM for OA. To modulate the susceptibility of tumor cells to lipid peroxidation, we used the oxidant mixture sodium ascorbate/2-methyl-1,4-naphthoquinone as a lipid peroxidation inducer and the anti-oxidant agent vitamin E as a lipid peroxidation inhibitor. The sodium ascorbate/2-methyl-1,4-naphthoquinone mixture at concentrations of 10^{-4} M/10^{-6} M or vitamin E at 10 μM induced no cytotoxic effect on MDA-MB-231 cells after 6 days of exposure (Fig. 1).

Addition of DHA to doxorubicin significantly decreased cell viability to 21 ± 1%. Addition of the oxidant mixture to cells supplemented with doxorubicin and DHA further decreased cell viability to 12 ± 1% (Fig. 1). Substitution of DHA by OA or substitution of oxidant agents by anti-oxidants caused cell viability to return to the baseline level of doxorubicin (Fig. 1).

To examine whether oxidative stress was involved, mitoxantrone was selected as a cytotoxic drug with low potential to generate free radical species and to promote lipid peroxidation. At 10^{-7} M mitoxantrone, no significant modification of cell viability was observed in any experiment under conditions identical to those employed with doxorubicin (data not shown).

Time course effect of DHA on doxorubicin cytotoxicity

To examine more precisely the effect of the combination of DHA with oxidant agents on the cytotoxicity of doxorubicin, cell viability was determined each day during 6 days of culture under different conditions (Fig. 2).

Doxorubicin at 10^{-7} M induced cell toxicity in a time-dependent manner since cell viability decreased from 99 ± 1% after 1 day to 49 ± 2% after 6 days of exposure. DHA enhanced significantly doxorubicin-induced cell toxicity after 6 days. With the DHA + oxidant mixture, the effect was detectable from 3 days of culture and became significant after 5 days in culture. The enhancing effect of DHA + oxidant was abolished when OA was used in place of DHA or when anti-oxidants were used in place of oxidants in the combined treatment (Fig. 2).

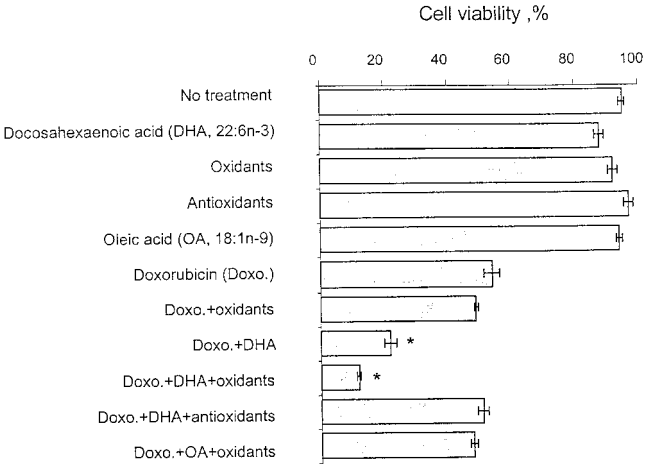

FIGURE 1 – Effect of DHA on doxorubicin-induced cell toxicity. MDA-MB-231 tumor cells were cultured for 6 days under different conditions in 24-well plates containing 10^{-7} M doxorubicin (Doxo.), oxidant agents (oxidants, sodium ascorbate/2-methyl-1,4-naphthoquinone, 10^{-4} M/10^{-6} M) or anti-oxidant (anti-oxidant, dl-α-tocopherol, 10^{-6} M), DHA (22:6n-3, 29 μM) or OA (18:1n-9, 34 μM). Cell viability was measured by Trypan blue exclusion after 6 days of exposure, and results represent mean ± SE of 3 separate experiments made in triplicate. *Significantly different from doxorubicin alone ($p < 0.05$, Mann-Whitney).

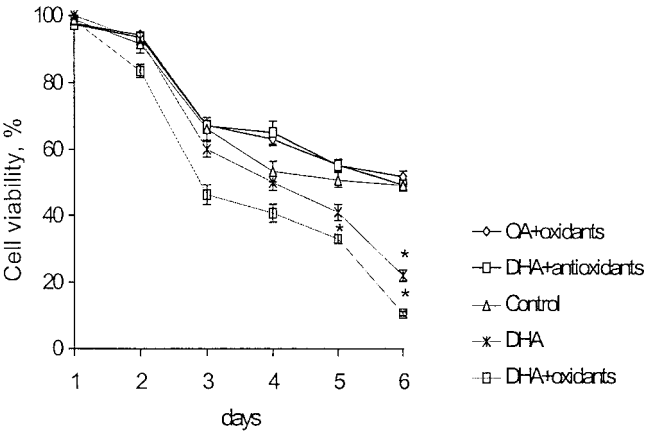

FIGURE 2 – Time course study of DHA on doxorubicin cytotoxicity under different culture conditions. MDA-MB-231 tumor cells were incubated for 6 days with 10^{-7} M doxorubicin either alone (control) or in the presence of DHA (22:6n-3, 29 μM) alone or with oxidant agents (sodium ascorbate/2-methyl-1,4-naphthoquinone, 10^{-4} M/10^{-6} M) or anti-oxidant (vitamin E, 10^{-6} M) or in the presence of OA (18:1n-9, 34 μM) with oxidant agents (sodium ascorbate/2-methyl-1,4-naphthoquinone, 10^{-4} M/10^{-6} M). Cell viability was determined every day by Trypan blue exclusion. Each point represents the mean ± SE of 3 separate experiments in which triplicate measurements were made. *Significantly different from experimental conditions with doxorubicin alone ($p < 0.05$, Mann-Whitney).

When mitoxantrone was used in place of doxorubicin, no modulating effect of DHA + oxidant was found at any time (data not shown).

Comparative effect of different PUFAs on doxorubicin-induced cell toxicity

The effects of other PUFAs with unsaturation degrees lower than DHA were examined on doxorubicin-induced cell toxicity. Experiments using eicosapentaenoic acid (20:5n-3), γ-linolenic acid (18:3n-6), α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) at non-cytotoxic concentration were carried out under conditions identical to those used with DHA (Fig. 3).

DHA was the most potent at enhancing the cytotoxic effect of doxorubicin (+70%), followed by γ-linolenic acid (+44%) and eicosapentaenoic acid (+18%). γ-Linolenic acid rendered cells more sensitive than did γ-linolenic acid ($p = 0.0005$). Oxidants enhanced the action of PUFAs on doxorubicin cytotoxicity: the effect was greater for eicosapentaenoic acid (47% *vs.* 18%) or arachidonic acid (45% *vs.* 11%) than for DHA (94% *vs.* 70%) or γ-linolenic acid (60% *vs.* 44%) with or without oxidants, respectively. Conversely, anti-oxidants abolished the action of PUFA on doxorubicin cytotoxicity since cell viability returned to the basal level induced by doxorubicin alone (Fig. 3).

Dose effect curve of doxorubicin and mitoxantrone in the presence of DHA and oxidants

The most effective combination of DHA (29 μM) + oxidant mixture (10^{-4} M sodium ascorbate/10^{-6} M 2-methyl-1,4-naphthoquinone) was used, and results are presented at 6 days of culture (Fig. 4).

Cell viability decreased from 65 ± 2% to 32 ± 2% at 3×10^{-8} M and from 75 ± 2% to 60 ± 2% at 10^{-8} M for doxorubicin alone or in the presence of DHA + oxidants. The calculated concentration of doxorubicin that induced 50% cell viability (IC_{50}) shifted from 0.79 to 0.13×10^{-7} M when doxorubicin was used in the presence of DHA + oxidants, a more than 5-fold increase in the sensitivity of MDA-MB-231 to doxorubicin (Fig. 4*a*). Such effects were not observed with mitoxantrone (Fig. 4*b*).

Lipid hydroperoxide level of breast tumor cells

The basal level of hydroperoxides in tumor cells was 8 pmol/μg proteins (Table I). Doxorubicin increased lipid hydroperoxide level in tumor cells by more than 2-fold. Addition of either DHA or oxidants to cells cultured with doxorubicin did not significantly change lipid hydroperoxide content in tumor cells. In contrast, DHA along with oxidant agents significantly increased lipid hydroperoxide content in tumor cells. A strong positive correlation

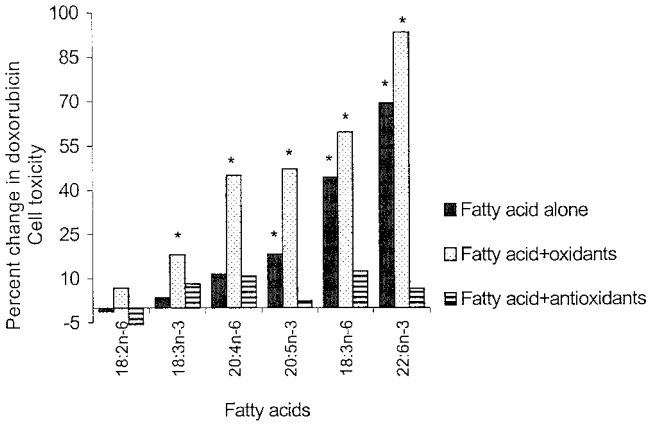

FIGURE 3 – Comparative effect of individual PUFAs on doxorubicin-induced cell toxicity. MDA-MB-231 tumor cells were cultured for 6 days as described in Figure 1. Cell toxicity of doxorubicin in the presence of specified PUFA alone or combined with oxidant agents (sodium ascorbate/2-methyl-1,4-naphthoquinone, 10^{-4} M/10^{-6} M) or anti-oxidant (dl-α-tocopherol, 10^{-6} M) was measured in 3 separate experiments performed in triplicate and compared to cell toxicity of 10^{-7} M doxorubicin alone (0% of variation = cell viability measured for condition with doxorubicin alone). Results expressed as percent of change of doxorubicin-induced cell toxicity. PUFAs are DHA (22:6n-3), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), γ-linolenic acid (18:3n-6), α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6). *Significantly different from experimental condition and doxorubicin alone ($p < 0.05$, Mann-Whitney).

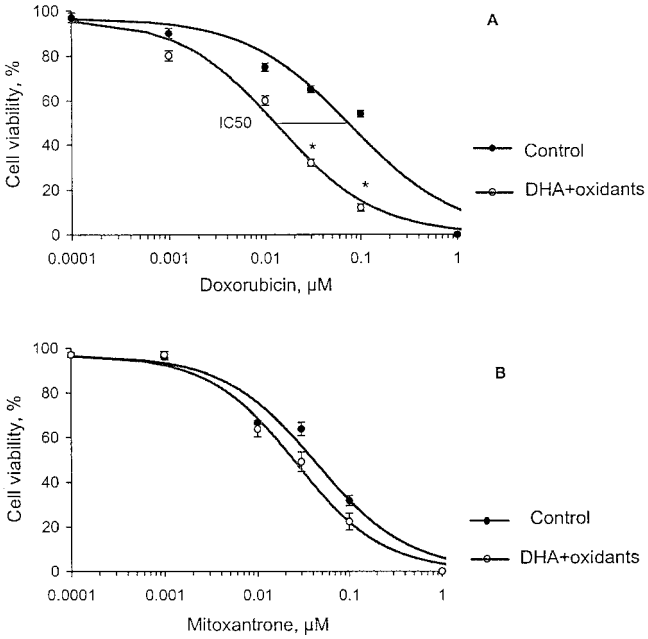

FIGURE 4 – Dose-response curve of doxorubicin and mitoxantrone in the presence of DHA and oxidant agents. MDA-MB-231 tumor cells were incubated with specified concentrations of doxorubicin *(a)* or mitoxantrone *(b)* either alone (Control) or in the presence of DHA + oxidant agents (sodium ascorbate/2-methyl-1,4-naphthoquinone) for 6 days. Cell viability was measured by Trypan blue exclusion. Shown are fitted curves and mean ± SE from 3 separate experiments in which triplicate measurements were made. *Significant difference between experimental condition and doxorubicin alone ($p < 0.05$).

was found between doxorubicin activity and the level of hydroperoxides in tumor cells (Fig. 5). Other conditions (substitution of oxidants by anti-oxidants or DHA by OA) led to no increase in hydroperoxide content (Table I).

When mitoxantrone was used in place of doxorubicin, no increase in hydroperoxide content was observed (Table I).

DISCUSSION

The ability of PUFAs to increase the cytotoxic activity of several anti-cancer drugs has been documented well. The involvement of lipid peroxidation as a potential mechanism through which PUFAs modulate the efficacy of those cytotoxic drugs has been suggested from experimental studies in tumor-bearing animals (Shao *et al.*, 1995) or in cell culture (Petersen *et al.*, 1992). In this report, we present data showing that PUFAs in conjunction with oxidants strongly increase the activity of a cytotoxic drug that generates an oxidative stress and that lipid peroxidation may be involved in this effect.

To explain the effect of PUFAs on the sensitivity of tumor cells to cytotoxic drugs, several mechanisms have been explored. Exogenous PUFAs are readily incorporated into breast cancer cell membranes (Goré *et al.*, 1994). Biochemical modifications of membrane fatty acids result in changes in the physical properties of cell membranes. This may lead to alteration in drug transport and, hence, may modulate cell sensitivity. Actually, the cellular concentration of doxorubicin increased in L1210 cells treated with DHA (Burns and North, 1986). Whether such a mechanism might be operating in the MDA-MB-231 cell line is not known.

To study lipid peroxidation as a mechanism through which PUFAs modulate the chemosensitivity of tumor cells, we selected 2 anti-cancer drugs which exhibit different potencies in inducing oxyradical formation: doxorubicin generates oxyradicals through its metabolism and subsequently induces peroxidative processes, whereas mitoxantrone does not (Doroshow, 1983). We found that doxorubicin increased lipid hydroperoxide content in tumor cells, while mitoxantrone did not. This finding is in agreement with previous studies carried out in subcellular fractions comparing lipid peroxidation stimulation (Kharasch and Novak, 1985; Vile and Winterboun, 1989) and in intact tumor cells comparing redox-

TABLE I – LIPID HYDROPEROXIDE CONTENT IN THE LIPID EXTRACT OF MDA-MB-231 TUMOR CELLS

Culture conditions	Hydroperoxides (pmol/µg proteins) mean ± SE
No treatment	7.9 ± 0.3
Doxorubicin (10^{-7} M)	17.4 ± 1.0[1]
Doxorubicin + DHA	22.5 ± 0.9[1]
Doxorubicin + oxidants	20.7 ± 1.4[1]
Doxorubicin + DHA + oxidants	31.1 ± 0.9[1,2]
Doxorubicin + DHA + anti-oxidants	14.5 ± 0.4[1]
Doxorubicin + OA + oxidants	14.0 ± 0.6[1]
Mitoxantrone (10^{-7} M)	6.3 ± 0.5
Mitoxantrone + DHA + oxidants	8.3 ± 0.8
Mitoxantrone + DHA + anti-oxidants	3.5 ± 0.3[1]
Mitoxantrone + OA + oxidants	7.6 ± 0.4

MDA-MB-231 cells were cultured in 6-well plates for 24 hr under specified conditions containing 10^{-7} M doxorubicin or 10^{-7} M mitoxantrone, oxidant agents (sodium ascorbate/2-methyl-1,4-naphthoquinone, 10^{-4} M/10^{-6} M) or anti-oxidants (dl-α-tocopherol, 10^{-6} M), DHA (29 µM) or OA (34 µM). The content of lipid hydroperoxides in the lipid extract of tumor cells was measured by an iodometric assay and protein content determined according to Lowry *et al.* (1951). In the absence of tumor cells, lipid hydroperoxides were undetectable in the medium containing 10^{-7} M doxorubicin and remained below 0.01 pmol/µg protein when DHA was added. Results are expressed as mean ± SE from 3 separate experiments in which duplicate measurements were made.–[1]Values significantly different from unsupplemented cells ($p < 0.05$, Mann-Whitney).–[2]Values significantly different between anti-cancer drugs and cells supplemented with drugs + different combined treatments ($p < 0.05$, Mann-Whitney).

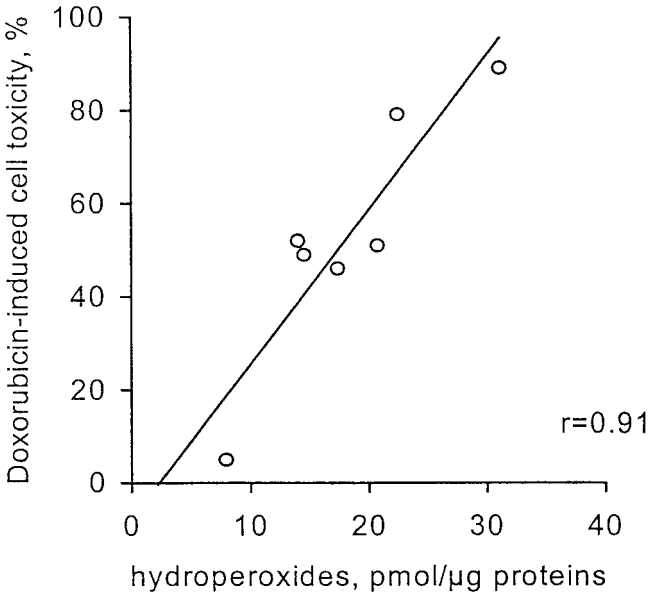

FIGURE 5 – Relationship between doxorubicin-induced cell toxicity and lipid hydroperoxide content in MDA-MB-231 breast tumor cells. Cell viability was estimated by Trypan blue exclusion after 6 days of exposure, and lipid hydroperoxide content was measured with the available cholesterol color reagent after 1 day of exposure, as described in ''Material and Methods''. Regression line with 95% confidence limits is shown.

cycling activation by these 2 drugs (Fisher and Patterson, 1992). Therefore, the comparison of the effect of PUFAs on doxorubicin *vs.* mitoxantrone efficacy in our model is suitable for the study of the involvement of oxidative stress and lipid peroxidation.

We examined the influence of different PUFAs on doxorubicin-induced cell toxicity. Among all methods available to assess drug activity, we chose to measure cell viability rather than cell proliferation. Although we did not evaluate cell clonogenicity, the Trypan blue exclusion test is less prone to pitfalls associated with the interpretation of cell proliferation assays. In agreement with previous studies (Burns and North, 1986; Zijlstra *et al.,* 1987), we observed that DHA was the most potent of all tested PUFAs at enhancing doxorubicin efficacy. For most of the PUFAs, the effect on drug activity increased with the double bond index. The only exception was 18:3n-6, which was more potent than 18:3n-3 and than fatty acids with 4 or 5 unsaturations (20:4n-6 or 20:5 n-3). This may be related to specific properties of 18:3n-6, as already documented in other cell lines (Bégin *et al.,* 1988).

We examined the involvement of lipid peroxidation as a potential mechanism by which PUFAs may modulate anti-cancer drug activity. Various methods are available for measuring the extent of lipid peroxidation. We chose to quantify hydroperoxide and hydroxy-PUFAs, which are the primary oxidation products in the lipid peroxidation process. We found that the increase in doxorubicin-induced cell death due to DHA was concomitant with an increase of lipid hydroperoxides. This suggests that lipid peroxidation could be involved. To further study the involvement of lipid peroxidation as a mechanism through which PUFAs modulate doxorubicin efficacy, we examined doxorubicin activity under conditions promoting or inhibiting lipid peroxidation. The potentiating effect exerted by PUFA alone was enhanced by conditions promoting lipid peroxidation: DHA along with an oxidant mixture was the most effective (*i*) at increasing doxorubicin cytotoxicity and (*ii*) at generating lipid hydroperoxides in breast tumor cells. We finally found that the sensitivity of MDA-MB-231 to doxorubicin was 5-fold higher in the presence of DHA along with oxidant agents. Conversely, in DHA-supplemented cells, substitution of the oxidant mixture by the anti-oxidant vitamin E abolished the effect and led both cell viability and lipid hydroperoxide content to return to baseline level. Thus, conditions favoring an increased lipid peroxidation in response to doxorubicin led to an increased activity of the drug. Conversely, inhibition of lipid peroxidation suppressed this additional activity of doxorubicin.

Although evidence suggests that lipid peroxidation could be involved in the cytotoxic activity of doxorubicin, the precise product of lipid peroxidation responsible presently is not known. Various data suggest a role for 4-hydroxyalkenal, an end-product of lipid peroxidation: transfection of CHO cells with a specific glutathione-*S*-transferase (GSTA4-4) which actively detoxifies one of the end-products of lipid peroxidation, including 4-hydroxynonenal, rendered transfected cells partially resistant to doxorubicin (He *et al.,* 1996). Similarly, 4-hydroxyalkenal by itself interferes with several vital cell functions (Esterbauer *et al.,* 1991). Moreover, lipid peroxides could act as mediators of programmed cell death (Sandstrom *et al.,* 1995). This result, in conjunction with our observations, supports the hypothesis that lipid peroxidation products may contribute to an additional cytotoxic activity of doxorubicin. In contrast to doxorubicin, we found that none of the tested conditions modulated mitoxantrone efficacy and lipid hydroperoxide cellular contents. These results strongly suggest that the cytotoxicity of mitoxantrone is not related to lipid peroxidation. The enhancing effect of PUFAs on anti-cancer drug activity may be restricted to drugs that induce oxidative stress.

In humans, little is known about the involvement of lipid peroxidation in drug efficacy. An increase in lipid peroxides measured after chemotherapy in plasma samples of patients with cancer has been reported (Faber *et al.,* 1995). Furthermore, in a study conducted on humans who had received neoadjuvant chemotherapy for locally advanced breast cancer, a positive correlation was found between DHA level in adipose breast tissue and subsequent sensitivity of the primary tumor to chemotherapy (Bougnoux *et al.,* 1995). Because fatty acids stored in the adipose tissue influence tumor cell membrane composition (Chajès *et al.,* 1995), this observation suggests that abundant targets for free radicals, such as highly unsaturated PUFAs, may generate lipid peroxidation products from the primary radicals after action of cytotoxic drugs. These products in turn could act as ''second cytotoxic messengers.'' Hence, cell death commitment signals could be locally strongly concentrated, leading to an enhanced tumor drug sensitivity and, therefore, to improved treatment efficacy. We are presently investigating the relevance of our observations *in vivo.* Our results suggest that a nutritional intervention based on highly unsaturated PUFAs along with pro-oxidants could improve the response of tumors to several anti-cancer agents in breast cancer patients. It remains to be determined to what extent the toxic side effects of these drugs (such as hematological or cardiac toxicity) would be affected. This condition is required before any intervention trial involving such a strategy can be considered in patients.

In conclusion, when the MDA-MB-231 cell line is cultured in the presence of DHA and oxidants, lipoperoxides arise after the action of oxyradical-producing anti-cancer drugs, such as doxorubicin. This may endow tumor cells with metabolic characteristics that decrease their propensity to survive the effects of doxorubicin. Whether PUFAs may modulate other anti-cancer drug activities and whether our observation might extend to other cell types remains to be investigated.

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