Tocotrienols and Human Breast Cancer

Tocotrienols were demonstrated to exert a direct inhibitory effect on the growth of human breast cancer cells. Tocotrienols offer a potential clinical application in the treatment of breast cancer patients.

Tocotrienols Inhibit the Growth of Human Breast Cancer Cells Irrespective of Estrogen Receptor Status

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ABSTRACT: Potential antiproliferative effects of tocotrienols, the major vitamin E component in palm oil, were investigated on the growth of both estrogen-responsive (ER+) MCF7 human breast cancer cells and estrogen-unresponsive (ER–) MDA-MB-231 human breast cancer cells, and effects were compared with those of α-tocopherol (αT). The tocotrienol-rich fraction (TRF) of palm oil inhibited growth of MCF7 cells in both the presence and absence of estradiol with a nonlinear dose-response but such that complete suppression of growth was achieved at 8 μg/mL. MDA-MB-231 cells were also inhibited by TRF but with a linear dose-response such that 20 μg/mL TRF was needed for complete growth suppression. Separation of the TRF into individual tocotrienols revealed that all fractions could inhibit growth of both ER+ and ER– cells and of ER+ cells in both the presence and absence of estradiol. However, the γ- and δ-fractions were the most inhibitory. Complete inhibition of MCF7 cell growth was achieved at 6 μg/mL of γ-tocotrienol/δ-tocotrienol ($\gamma T_3/\delta T_3$) in the absence of estradiol and 10 μg/mL of δT_3 in the presence of estradiol, whereas complete suppression of MDA-MB-231 cell growth was not achieved even at concentrations of 10 μg/mL of δT_3. By contrast to these inhibitory effects of tocotrienols, αT had no inhibitory effect on MCF7 cell growth in either the presence or the absence of estradiol, nor on MDA-MB-231 cell growth. These results confirm studies using other sublines of human breast cancer cells and demonstrate that tocotrienols can exert direct inhibitory effects on the growth of breast cancer cells. In searching for the mechanism of inhibition, studies of the effects of TRF on estrogen-regulated pS2 gene expression in MCF7 cells showed that tocotrienols do not act *via* an estrogen receptor-mediated pathway and must therefore act differently from estrogen antagonists. Furthermore, tocotrienols did not increase levels of growth-inhibitory insulin-like growth factor binding proteins (IGFBP) in MCF7 cells, implying also a different mechanism from that proposed for retinoic acid inhibition of estrogen-responsive breast cancer cell growth. Inhibition of the growth of breast cancer cells by tocotrienols could have important clinical implications not only because tocotrienols are able to inhibit the growth of both ER+ and ER– phenotypes but also because ER+ cells could be growth-inhibited in the presence as well as in the absence of estradiol. Future clinical applications of TRF could come from potential growth suppression of ER+ breast cancer cells otherwise resistant to growth inhibition by antiestrogens and retinoic acid.

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Tocopherols and tocotrienols, collectively known as vitamin E, are lipid-soluble compounds that are often present as natural components in vegetable oils. Structurally, these compounds are similar, except that tocotrienols have an unsaturated side chain with three double bonds whereas the tocopherols have a fully saturated side chain (1). In their function as antioxidants, they are important for the protection of unsaturated lipids against peroxidation, particularly in biomembranes (2), by a mechanism resulting mainly from their ability to donate phenolic hydrogens to lipid-free radicals (3–5). Whereas the vitamin E fraction of most common vegetable oils contains mainly tocopherols, palm oil is a rich source of tocotrienols, with refined palm oil containing 133 mg α-tocopherol (αT), 130 mg α-tocotrienol (αT_3), 204 mg γ-tocotrienol (γT_3), and 45 mg δ-tocotrienol (δT_3) per kg of oil (6).

Experimental studies both *in vitro* and *in vivo* have suggested that tocotrienols may possess anticancer properties (7,8). Unlike many other fats and oils, palm oil does not enhance the yield of chemically-induced mammary tumors when fed to rats at high levels (20% w/w fat) in a semipurified diet (9–11). Evidence that this effect is related to the vitamin E fraction of the palm oil [tocotrienol-rich fraction (TRF)] was shown in our recent study (12) where chemically-induced mammary tumors were more numerous in rats fed vitamin E-free palm oil than in those animals fed palm oil containing vitamin E. Furthermore, addition of the vitamin E fraction of palm oil to a corn oil diet increased the median latency period and reduced both tumor incidence and tumor yield in a dose-dependent manner (12).

This inhibitory action of tocotrienols on breast tumor growth *in vivo* could result either from direct effects on the growth of the tumor cells themselves or from indirect sys-

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Abbreviations: DCFCS, dextran-charcoal treated fetal calf serum; ER+, estrogen receptor positive (containing estrogen receptors); ER–, estrogen receptor negative (lacking estrogen receptors); FCS, fetal calf serum; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; αT, α-tocopherol; αT_3, α-tocotrienol; γT_3, γ-tocotrienol; δT_3,δ-tocotrienol; TRF, tocotrienol-rich fraction.

temic interactions. Studies on the growth of MDA-MB-435 human breast cancer cells *in vitro* have demonstrated that the TRF of palm oil can inhibit directly the growth of these cells whereas αT did not have any such effect (13). However, the MDA-MB-435 cells lack estrogen receptors, and estrogen is known to play an important role in the growth of many breast cancer cells *in vivo* (14,15) and *in vitro* (16,17). Thus, the work presented here extends these cell culture studies to compare the effects of tocotrienols on the growth of human breast cancer cells which possess estrogen receptors and which are estrogen-responsive for growth with those cells which lack estrogen receptors.

Growth of the ER+ MCF7 human breast cancer cell line is regulated *in vitro* by estrogens (16,18) and offers a suitable model system in which to study the effects of tocotrienols. Separate sublines of MCF7 cells show different sensitivities to estrogen for growth, but the MCF7McGrath subline (19) is so dependent on estrogen for growth that it is unable to proliferate to any large extent in the absence of estrogen (16). However, recent studies have shown that growth of these cells is regulated also by complex interactions with growth factors of which one major mitogenic component would appear to be the insulin-like growth factors (IGF) (17,20). Growth regulation by the IGF system is itself complex since there are two ligands (IGFI and IGFII), two receptors (IGFIR and IGFIIR), and at least six high-affinity extracellular binding proteins (IGFBP 1-6) (21), and it would appear that estrogen can alter the expression of several of these components in breast cancer cells including IGFII (22), IGFIR (23), and IGFBP (24). Growth inhibitory effects in estrogen-responsive human breast cancer cells could thus result either from a direct antagonism of estrogen action at the estrogen receptor level (25) as found for antiestrogens such as tamoxifen and ICI 182,780 (26,27) or from interactions in growth factor pathways such as changing levels of IGFBP (28). The work presented here describes the effects of the TRF of palm oil and of individual tocotrienols on the growth of estrogen-responsive MCF7McGrath human breast cancer cells, on pS2 gene expression as a molecular marker of estrogen action (29), and on levels of secreted IGFBP.

MATERIALS AND METHODS

Materials. Plastic tissue culture dishes, growth media, and fetal calf serum (FCS) were all purchased from Gibco BRL (Paisley, Scotland). Zaponin and isoton were purchased from Coulter Electronics (Harpenden, England). Hybond membranes were bought from Amersham International (Amersham, England). 17-β-Estradiol was purchased from Steraloids (Croydon, England). The TRF and individual αT_3, γT_3, and δT_3 fractions were obtained from the Palm Oil Research Institute of Malaysia (PORIM). The αT was obtained from Sigma Chemical Company (Croydon, England) at a purity of approximately 95%.

Isolation of TRF and individual tocotrienols. Extraction of the TRF from palm oil has been described by Sundram and Gapor (30). In brief, palm oil fatty acid distillate was converted into methyl esters by esterification. The methyl esters were then removed by distillation, leaving a vitamin E concentrate. This was further concentrated by crystallization and passed through an ion-exchange column to give 60–70% pure vitamin E. Further purification was achieved by washing and then drying the concentrate followed by a second molecular distillation stage. The final purity of the vitamin E preparation, TRF, was 95–99%, its composition being (w/w) αT 32%, αT_3 25%, γT_3 29%, and δT_3 14%.

Individual tocotrienols were separated by thin-layer chromatography and further chromatographed repeatedly until a purity of about 90% (highest available) was obtained.

Culture of stock cells. The MCF7 McGrath human breast cancer cells were kindly provided by Dr. K. Osborne at passage number 390 (19). Stock cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS, 10^{-8} M estradiol, and 10 μg/mL insulin in a humidified atmosphere of 10% carbon dioxide in air at 37°C. 17-β-Estradiol was dissolved in ethanol and diluted 1:10,000 in culture medium. Insulin was dissolved in 6 mM HCl and diluted 1:1,000 in culture medium. MDA-MB-231 human breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA). Stock cells were grown as for MCF7 cells but with the omission of estradiol and insulin. Cells were subcultured at weekly intervals by suspension with 0.06% trypsin/0.02% EDTA (pH 7.3).

Cell growth experiments. Cells were suspended from stock plates by treatment with phenol red-free 0.06% trypsin/0.02% EDTA (pH 7.3), added to an equal volume of phenol red-free RPMI1640 medium containing 5% dextran-charcoal treated FCS (DCFCS) (31), and counted on a hemacytometer. Cells were then added to the required volume of phenol red-free RPMI1640 medium containing 5% DCFCS at a concentration of 0.2×10^5 cells/mL and plated in monolayer in 0.5-mL aliquots into 24-well plastic tissue culture dishes. After 24 h, the medium was changed to phenol red-free RPMI1640 medium with 5% DCFCS supplemented with the appropriate concentrations of TRF, αT, individual tocotrienol fractions, with or without estradiol. Both TRF and αT were dissolved in dimethyl sulfoxide, estradiol in ethanol, and all were diluted 1:10,000 in culture medium. Control cultures contained the same volume of dimethyl sulfoxide or ethanol vehicle alone. The culture medium was changed routinely every 3–4 d.

Cell counting. Cells were washed rigorously *in situ* with phosphate-buffered saline (PBS) in order to wash off nonadherent dead cells and were then lysed in 0.5 mL 0.01 M HEPES buffer/1.5 mM $MgCl_2$ plus 2 drops of zaponin solution for 5 min. The nuclei released were counted in isoton on a Coulter counter. All cell counts were carried out in triplicate on triplicate dishes and results calculated as the mean ± standard error. *P* values were determined using Student's *t*-test for two-samples assuming unequal variance [by standard software packages Minitab and Microsoft Excel (Microsoft Corp., Redmond, WA)]. Viability of adherent cells was determined using the standard try-

pan blue dye exclusion assay technique and performing cell counts on a hemacytometer.

RNA analysis by Northern blotting. Cells were plated onto 9-cm plastic tissue culture dishes in 16-mL aliquots of phenol red-free RPMI1640 medium with 5% DCFCS. After 24 h, the medium was changed to phenol red-free RPMI1640 medium containing 5% DCFCS with or without 10^{-8} M estradiol or 8 μg/mL TRF. After a further 6 d, cells were washed *in situ* with PBS, harvested into ice-cold PBS using a rubber policeman and pelleted by centrifugation. Whole cell RNA was prepared by the guanidinium cesium chloride method (32). Aliquots of RNA (20 μg) were subjected to electrophoresis in 1.5% agarose-formaldehyde gels (32). RNA was transferred onto Hybond-N membrane and hybridized to ^{32}P-labeled cDNA probes for pS2 and 36B4 exactly as described previously (29). The pS2 DNA probe was a 300-base pair P*st*1 fragment from pS2 cDNA (29). The 36B4 DNA probe was a 220-base pair P*st*1 fragment which acted as a control since 36B4 mRNA is not regulated by estrogen (33).

Western ligand blotting of IGFBP. Cells were plated onto 3.5-cm plastic tissue culture dishes in 2.5-mL aliquots of phenol red-free RPMI1640 medium supplemented with 5% DCFCS. After 24 h, the medium was changed to phenol red-free RPMI1640 medium with 5% DCFCS and supplemented with 10^{-8} M estradiol or with 6 μg/mL of αT, αT_3, γT_3, or δT_3 as required. After a further 6 d, the medium was changed to serum-free medium: cells were washed three times with phenol red-free RPMI1640 medium and incubated in 0.5 mL of serum-free medium per dish (phenol red-free RPMI1640 medium with 15 mM HEPES buffer, 0.25% bovine serum albumin, and any supplements of estradiol or vitamin E as above) for a further 24 h. Medium conditioned by the cells was collected, cellular material removed by centrifugation, and medium stored at –70°C. Cells remaining on each dish were counted as described above.

Aliquots of the conditioned media were run on polyacrylamide gel electrophoresis, loading into each well the volume of conditioned medium equivalent to 10^5 cells. Aliquots of conditioned media were each mixed with an equal volume of gel sample buffer (26 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue), heated to 100°C for 2 min, and proteins separated by 15% polyacrylamide-SDS gel electrophoresis. Proteins were transferred onto Hybond-C extra membrane by semidry Western blotting in 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol. Western blots were hybridized to ^{125}I-IGFI as described by Hossenlopp and coworkers (34). ^{125}I-IGFI was prepared by the iodogen method (35).

RESULTS

Effect of TRF and αT on cell growth. Initial experiments were designed to determine the effect of TRF on the growth of MCF7McGrath human breast cancer cells. Figure 1A shows

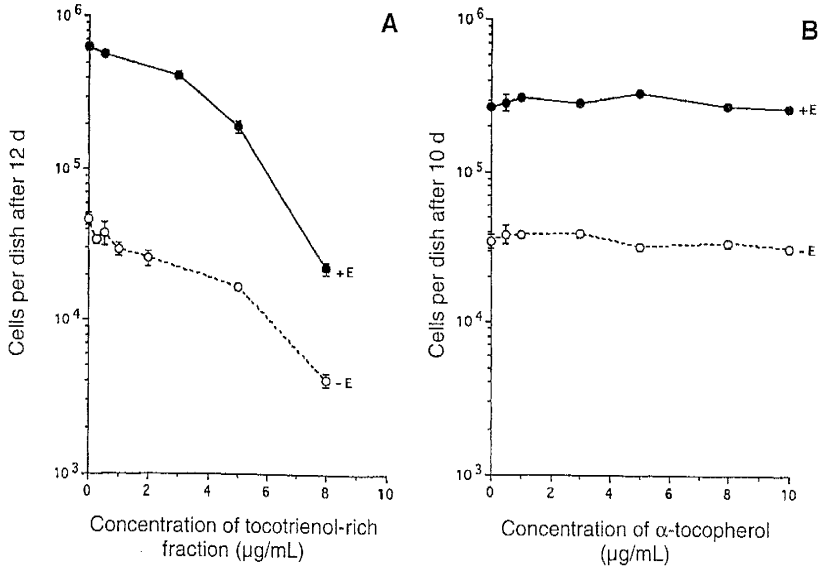

FIG. 1. Effect of the tocotrienol-rich fraction (TRF) of palm oil (A) or α-tocopherol (B) on regulation of the growth of estrogen-responsive ER+ MCF7McGrath human breast cancer cells in monolayer culture. Cells were grown for (A) 12 d or (B) 10 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% dextran-charcoal treated fetal calf serum (DCFCS) and increasing concentrations of either TRF or α-tocopherol (αT) in either the absence (open circles and dotted lines) or presence (closed circles and solid lines) of 10^{-8} M estradiol. Plating densities of the cells were (A) $1.34 \pm 0.01 \times 10^4$ cells per dish for the experiment in the absence of estradiol, and $2.41 \pm 0.08 \times 10^4$ cells per dish for the experiment in the presence of estradiol; (B) $0.43 \pm 0.02 \times 10^4$ cells per dish for the experiment in the absence of estradiol, and $0.64 \pm 0.01 \times 10^4$ cells per dish for the experiment in the presence of estradiol. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display.

that TRF inhibited the growth of these estrogen-responsive cells both in the absence and in the presence of estradiol, and in a dose-dependent manner. On its own, TRF inhibited cell growth at concentrations of 0.5 μg/mL ($P = 0.031$), 1 μg/mL ($P = 0.04$), 2 μg/mL ($P = 0.027$), 5 μg/mL ($P = 0.018$), and 8 μg/mL ($P = 0.009$). At 8 μg/mL TRF, cell numbers at 12 d fell below the plating density of the cells ($P = 0.002$). In the presence of 10^{-8} M estradiol, TRF was also able to inhibit cell growth at concentrations of 0.5 μg/mL ($P = 0.04$), 3 μg/mL ($P = 0.01$), 5 μg/mL ($P = 0.002$), and 8 μg/mL ($P = 0.005$), but cell numbers remained above plating density in all cases. In contrast to these results with TRF, Figure 1B shows that αT had no effect on growth of MCF7McGrath human breast cancer cells at any concentration tested from 0.5–10.0 μg/mL in either the absence or in the presence of 10^{-8} M estradiol (after 10 d at 10 μg/mL αT, in the absence of estradiol $P = 0.421$ and in the presence of estradiol $P = 0.535$).

These results are in line with previous studies using ER– MDA-MB-435 human breast cancer cells (13) where cell growth was similarly shown to be inhibited by TRF but not by αT. However, since these previous studies were based on different cell biological criteria, control experiments were repeated here using ER– MDA-MB-231 human breast cancer cells for direct comparison. Figure 2A shows that after 10 d, TRF inhibited cell growth at concentrations of 2 μg/mL ($P = 0.003$), 5 μg/mL ($P = 0.002$), 8 μg/mL ($P = 0.001$) and 10 μg/mL ($P = 0.0001$) but in all cases cell growth remained substantial and cell numbers well above plating density. However, at 20 μg/mL, TRF was able to inhibit MDA-MB-231 cell growth and reduce cell numbers below plating density ($P = 0.057$) (data not shown). In contrast, αT had no effect on growth of MDA-MB-231 cells at any concentration up to 10 μg/mL (Fig. 2B) or indeed even at 20 μg/mL (data not shown).

Effect of individual tocotrienols on cell growth. Further experiments studied the effects on cell growth of individual tocotrienol fractions (α-, γ-, and δ-) in order to determine which of the fractions were effective or most effective in inhibiting the cell growth. These results are shown in Figures 3 and 4. For MCF7McGrath cells, γT_3 and δT_3 were the most effective of the fractions at inhibiting cell growth in the absence of estradiol (Fig. 3) and did so in a dose-dependent manner after both 7 and 14 d in culture. Already after 7 d, γT_3 showed highly significant inhibitory effects at concentrations from 4 μg/mL ($P = 0.001$) to 6 μg/mL ($P = 0.002$). At 6 μg/mL γT_3, cell growth was completely suppressed with cell numbers even falling below the plating density ($0.130 \pm 0.086 \times 10^5$ cells on day 0; $0.057 \pm 0.002 \times 10^5$ cells on day 7; $0.021 \pm 0.076 \times 10^5$ cells on day 14). δT_3 also inhibited cell growth after 7 d at concentrations from 4 μg/mL ($P = 0.03$) to 6 μg/mL ($P = 0.007$). Again, at 6 μg/mL δT_3, cell growth was completely suppressed with cell numbers even falling below the plating density ($0.078 \pm 0.005 \times 10^5$ cells on day 0; $0.069 \pm 0.046 \times 10^5$ cells on day 7; $0.040 \pm 0.014 \times 10^5$ cells on day 14). αT_3 had no inhibitory effect on growth of MCF7McGrath cells after 7 d in culture (at 4 μg/mL $P = 0.569$; at 5 μg/mL $P = 0.482$; at 6 μg/mL $P = 0.249$) and had only a small inhibitory effect after 14 d in culture at concentrations of 5 μg/mL ($P = 0.038$) and 6 μg/mL ($P = 0.022$).

In the presence of estradiol, MCF7McGrath cell growth was inhibited most strongly by δT_3 (Fig. 3) (after 14 d, *P* val-

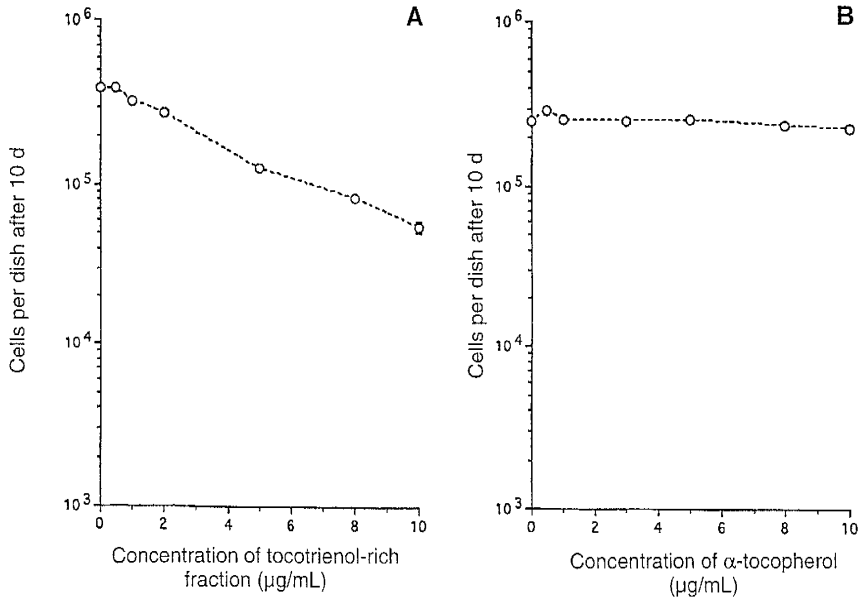

FIG. 2. Effect of the tocotrienol-rich fraction (TRF) of (A) palm oil or (B) αT on regulation of the growth of estrogen-insensitive ER– MDA-MB-231 human breast cancer cells in monolayer culture. Cells were grown for 10 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of either TRF or αT in the absence of estradiol. Cells were plated together in one experiment for A and B at a density of $1.05 \pm 0.19 \times 10^4$ cells per dish. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviations.

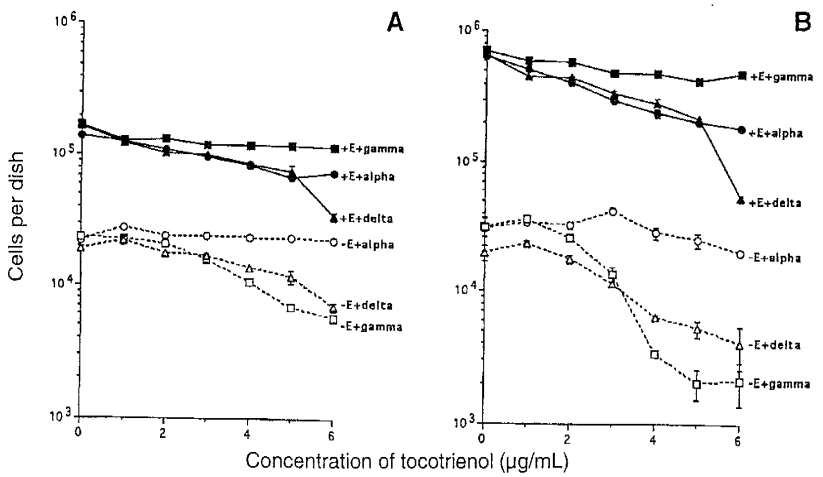

FIG. 3. Effect of individual tocotrienols on regulation of the growth of estrogen-responsive ER+ MCF7McGrath human breast cancer cells in monolayer culture. Cells were grown for (A) 7 or (B) 14 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of α-tocotrienol (αT_3) (●,○), γ-tocotrienol (γT_3) (■,□), or δ-tocotrienol (δT_3) (▲,△) in either the absence of estradiol (–E) (open symbols, dotted lines) or the presence of 10^{-8} M estradiol (+E) (closed symbols, solid lines). Cells were plated at a density of $1.06 \pm 0.08 \times 10^4$ cells per dish for the $-E + \alpha T_3$ experiment, of $1.30 \pm 0.08 \times 10^4$ cells per dish for the $-E + \gamma T_3$ experiment, of $0.78 \pm 0.05 \times 10^4$ cells per dish for the $-E + \delta T_3$ experiment, and of $2.44 \pm 0.21 \times 10^4$ cells per dish for the entire +E experiment with αT_3, γT_3, and δT_3. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display.

ues were 1 μg/mL $P = 0.009$; 2 μg/mL to 6 μg/mL $P < 0.001$). At a concentration of 10 μg/mL (data not shown), all three fractions could inhibit cell growth after 7 d (αT_3 by 63%, γT_3 by 32%, δT_3 by >100%) and after 14 d (αT_3 by 61%, γT_3 by 14%; δT_3 by >100%). At 10 μg/mL, δT_3 reduced cell numbers below plating density ($P = 0.008$ after 7 d; $P = 0.001$ after 14 d).

Parallel comparisons using ER– MDA-MB-231 cells showed that cell growth could be inhibited by all three tocotrienol fractions but most markedly by the δT_3 (Fig. 4). However, even at concentrations of 10 μg/mL (data not shown), cell numbers did not fall below plating density.

Cell viability. Where cell numbers in monolayer culture fell below plating density implying cell death, trypan blue vi-

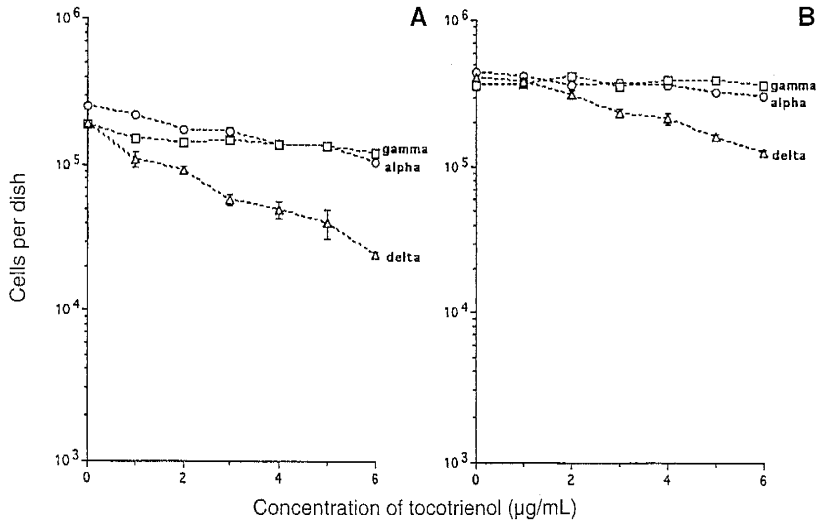

FIG. 4. Effect of individual tocotrienols on regulation of the growth of estrogen-insensitive ER– MDA-MB-231 human breast cancer cells in monolayer culture. Cells were grown for (A) 7 or (B) 14 d in 24-well dishes in RPMI1640 medium lacking phenol red but containing 5% DCFCS and increasing concentrations of αT_3 (●,○), γT_3 (■,□), or δT_3 (▲,△) in the absence of estradiol. Cells were plated together for the entire experiment at a density of $0.99 \pm 0.08 \times 10^4$ cells per dish. Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviation.

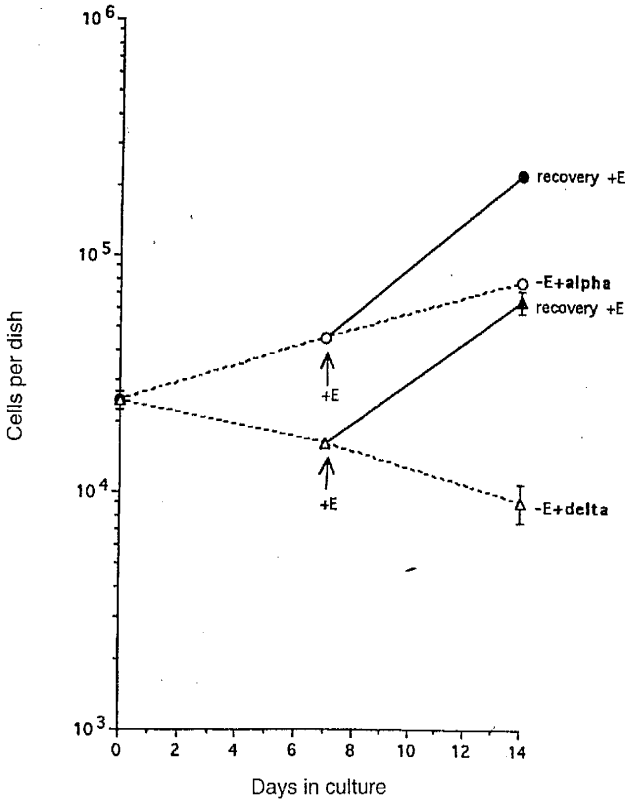

FIG. 5. Recovery of MCF7McGrath cell growth following a 7-d exposure to αT_3 or δT_3. MCF7McGrath human breast cancer cells were grown in monolayer culture for 14 d in 24-well dishes in RPMI1640 medium lacking both phenol red and estradiol but containing 5% DCFCS and either 6 μg/mL αT_3 (–E + alpha) (open circles, dotted lines) or 6 μg/mL δT_3 (–E + delta) (open triangles, dotted lines). Parallel dishes of cells were grown as above for 7 d but then for a further 7 d in RPMI1640 medium with 5% DCFCS and 10^{-8} M estradiol without tocotrienol (solid circle, solid line for 7 d with αT_3, 7 d recovery with estradiol; solid triangle, solid line for 7 d with δT_3, 7 d recovery with estradiol). Error bars represent the standard error of triplicate dishes. Where no bars are seen, the error was too low for visual display. See Figure 1 for abbreviation.

ability counts were carried out in order to ascertain the viability of remaining adherent cells. MCF7McGrath human breast cancer cells grown without estradiol but with 10 μg/mL TRF showed a viability count of 98.8 ± 1.2% for cells remaining attached to the dish after 12 d (cf. Fig. 1A). Growth of MCF7 cells with 6 μg/mL of individual tocotrienol fractions in the absence of estradiol (cf. Fig. 3) gave viability counts after 7 d of 91.9 ± 2.4% for αT_3, 89.1 ± 1.3% for γT_3, 94.3 ± 3.1% for δT_3, and after 14 d of 78.4 ± 4.1% for αT_3, 72.0 ± 2.7% for γT_3, 73.9 ± 3.9% for δT_3. To test further for the viability of remaining adherent cells, a recovery experiment was performed in which cells were grown for 7 d with 6 μg/mL of either αT_3 or δT_3 followed by a further 7-d recovery period with estradiol. The results shown in Figure 5 demonstrate that cells exposed for 7 d to tocotrienol were subsequently still capable of regrowth in monolayer culture when tocotrienol was removed and estradiol added back.

Regulation of pS2 mRNA. In order to investigate any estrogen antagonist properties of TRF, effects of TRF were studied on pS2 mRNA levels. pS2 is an estrogen-regulated gene whose expression can be inhibited at the estrogen receptor level by antiestrogens and estrogen antagonists (29). At the concentrations tested, TRF (8 μg/mL) did not reduce the levels of pS2 mRNA either in the presence or in the absence of estradiol (Fig. 6). 36B4 mRNA is not regulated by estrogen, and levels of this mRNA are given to control for unequal loading of RNA samples. When comparisons were made of relative levels of pS2 mRNA to 36B4 mRNA, TRF was found to have no effect on pS2 mRNA levels either in the presence or in the absence of estradiol.

Regulation of IGFBP. Levels of IGFBP have also been shown to be altered by estrogen in estrogen-responsive human breast cancer cells, and this may have a functional role in growth regulation (24). Results given in Figure 7 show that IGFBP2 (32 kDa) and IGFBP4 (24 kDa) are the two major binding proteins secreted from MCF7McGrath cells, as reported previously (36). Levels of these IGFBP were not increased by γT_3 or δT_3.

DISCUSSION

The ability of the TRF of palm oil to inhibit mammary carcinogenesis in animal studies (5–12) is of potential importance for cancer prevention and treatment, but the mechanism remains unresolved even to the extent of knowing whether the action is directly on the tumor cells or indirectly *via* systemic interactions. The results of this study demonstrate that the TRF of palm oil can inhibit the growth of estrogen-responsive MCF7McGrath as well as estrogen-insensitive MDA-MB-231 human breast cancer cells *in vitro* and furthermore that MCF7 cell growth can be inhibited by TRF irrespective of the presence or absence of estradiol. These data confirm other recent studies using other sublines of ER– (13,37) and ER+ (38–40) breast cancer cells and suggest that the inhibition of tumor growth found *in vivo* could result, at least in part, from direct inhibitory effects on the growth of the cancer cells.

Comparison between growth of MCF7 and MDA cells shows that the inhibitory growth responses to tocotrienols differ in the two cell lines. Growth of MDA-MB-231 cells gave an inhibitory response which was linear with respect to increasing concentrations of tocotrienol whereas the MCF7 cell response was nonlinear. Thus, tocotrienols have greater inhibitory effects at low concentration on the MDA cells but at higher concentrations on the MCF7 cells. The 10-d growth of MDA cells (Fig. 2) could be suppressed by one doubling (3.96×10^5 to 1.98×10^5 cells/dish) by 3 μg/mL TRF, whereas 12-d growth of MCF7 cells (Fig. 1) in the presence of estradiol required 4 μg/mL TRF for suppression by one doubling (6.29×10^5 to 3.15×10^5 cells/dish). However, complete suppression of growth was achieved at lower concentration of TRF for the MCF7 cells. The growth of the MCF7 cells showed 100% growth inhibition with 8 μg/mL of TRF irrespective of the presence of estradiol, whereas the MDA cells

showed only 44% growth inhibition at this concentration, requiring 20 μg/mL TRF for 100% growth inhibition. Separation of the TRF into individual tocotrienols α-, γ- and δ- revealed that the γ- and δ-fractions were the most inhibitory to breast cancer cell growth and again showed the same differences in response in the two cell lines. The 7-d growth of MDA-MB-231 cells (Fig. 4A) could be suppressed by one doubling (1.95×10^5 to 0.98×10^5 cells/dish) by 2 μg/mL δT_3, whereas 7-d growth of MCF7 cells (Fig. 3A) in the presence of estradiol needed 5 μg/mL δT_3 for suppression by one doubling (1.66×10^5 to 0.83×10^5 cells/dish). However, complete suppression of MCF7 cell growth was achieved at concentrations of 6 μg/mL $\gamma T_3/\delta_3$ in the absence of estradiol and at 10 μg/mL δT_3 in the presence of estradiol, whereas for MDA-MB-231 cell growth complete suppression of growth was never achieved even at 10 μg/mL concentrations of the most inhibitory δT_3 component.

It is interesting that TRF (8 μg/mL) and $\gamma T_3/\delta T_3$ (6 μg/mL) could inhibit MCF7McGrath cell growth such that cell numbers were decreased below the plating density since this implies cell death. It would now be interesting therefore to determine whether tocotrienols can induce apoptotic pathways in the ER+ cells, as has already been described in breast cancer cells for hormone ablation (41), antiestrogen treatment (42,43), antagonism of IGF pathways (44), and administration of other cytotoxic agents (45,46).

Although it is often stated that αT is the most active form of vitamin E in inhibiting growth of cells (47), none of the breast cancer cells showed any sensitivity to αT in either these or other (13,39) studies. This emphasizes the importance of tocotrienols to growth inhibition of breast cancer cells. The reasons for the high sensitivity of breast cancer cells to tocotrienols are unknown but could relate either to permeability of the breast epithelial cell membrane to tocotrienols or to metabolism of tocotrienol within the breast cells (13). Alternatively, there may prove to be a pathway important specifically for breast cancer cell proliferation which is highly sensitive to tocotrienols. Inhibition of smooth muscle cell proliferation by vitamin E has been suggested to occur through alteration of protein kinase C activity (47) which could result from the antioxidant properties of vitamin E (48). Tocotrienols have been reported to affect lipid parameters, in particular linoleic acid metabolism (49), and it is known that linoleic acid plays a role in mammary epithelial cell growth and differentiation (50; unpublished personal data). Within estrogen-responsive breast cancer cells, another potential pathway of growth inhibition could be through antagonism of estrogen action, in a mechanism analogous to that characterized for growth inhibition by antiestrogens (25–27). Estrogen is known to act *via* an intracellular receptor protein which functions as a ligand-activated transcription factor (25), and it is now well established that the partial antiestrogen tamoxifen can act by interfering with transcriptional activation by the estrogen receptor and that the pure antiestrogens ICI 164,384 and ICI 182,780 can act by preventing dimerization and DNA binding of estrogen receptors (26,27). The TRF of

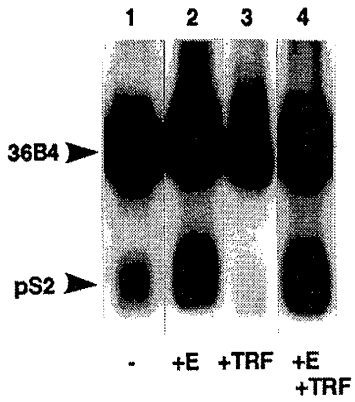

FIG. 6. Effect of the tocotrienol-rich fraction (TRF) of palm oil on expression of pS2 mRNA in MCF7McGrath human breast cancer cells. Northern blot of whole cell RNA from cells grown for 6 d in monolayer culture with 5% DCFCS alone (–) (track 1), with 10^{-8} M estradiol (+E) (track 2), with 8 μg/mL TRF (+TRF) (track 3) or with both 10^{-8} M estradiol and 8 μg/mL TRF (+E + TRF) (track 4). The blot was probed for expression of the estrogen-regulated pS2 mRNA and also a control non-estrogen-regulated 36B4 mRNA to control for any unequal RNA loadings. Positions of the mRNA are indicated by arrows. See Figure 1 for other abbreviation.

palm oil was clearly able, in the present studies, to inhibit growth of the MCF7McGrath cells in the presence of estrogen. In the absence of estrogen, TRF could also have acted to antagonize the action of residual estrogen in the cell or estrogen memory effects (51). However, TRF appeared to be unable to alter expression of the estrogen-regulated pS2 gene in MCF7McGrath cells, which is a well-established marker of estrogen receptor-mediated action (29) (Fig. 6). Furthermore, if TRF were acting on cell growth in the absence of estrogen to inhibit residual estrogen effects, it would be expected that TRF would act in a concentration-dependent manner such that lower levels of TRF would be needed to inhibit growth in the absence than in the presence of estrogen. Clearly this was not the case since inhibition of MCF7McGrath cell growth was found to require similar concentrations of TRF in the absence and in the presence of estrogen. The overall evidence would suggest therefore that tocotrienols do not act by an estrogen receptor-mediated mechanism.

An alternative mechanism for growth inhibition by TRF could relate to IGFBP (Fig. 7). Recent reports have emphasized the importance not only of estrogen but also of the insulin-like growth factors in breast cancer cell growth (20). IGFBP can influence the interaction between IGF and their receptors and so play an important role in mediating IGF regulation of breast cancer cell growth (20). It is thus possible that TRF could inhibit breast cancer cell growth by increasing production of inhibitory IGFBP in an analogous mechanism to that proposed for growth inhibition by retinoic acid in breast cancer cells (52). The growth inhibitory γT3 and δT3, however, did not appear to increase levels of IGFBP in these studies.

Inhibition of the growth of breast cancer cells by tocotrienols could have important clinical implications not only

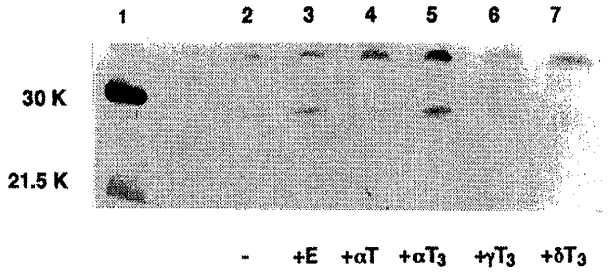

FIG. 7. Effect of individual tocotrienols on the secretion of insulin-like growth factor binding protein (IGFBP) from MCF7McGrath human breast cancer cells. Western ^{125}I-IGFI ligand blot of cells grown for 6 d in monolayer culture in RPMI1640 medium with 5% DCFCS alone (–) (track 2), with 10^{-8} M estradiol (+E) (track 3), with 6 μg/mL +αT (track 4), with 6 μg/mL αT_3 (track 5), with 6 μg/mL γT_3 (track 6), or with 6 μg/mL δT_3 (track 7). Positions of ^{14}C-labeled protein molecular weight markers are given in track 1 and sizes in kDa are indicated on the left-hand side. In tracks 2–7, the upper band corresponds to IGFBP2 (32 KDa) and the lower band to IGFBP4 (24 KDa) as reported previously (36). See Figure 1 for other abbreviation.

because tocotrienols are able to inhibit the growth of both ER+ and ER– phenotypes but also because estrogen-responsive cells could be inhibited in the presence as well as in the absence of estrogen. Unfortunately the molecular mechanism of the growth inhibition remains unknown and will need further work to elucidate. However, from a clinical perspective, it will also be of interest to determine whether breast cancer cells can develop resistance to this pathway of growth inhibition by TRF. A major problem in the therapy of breast cancer results from the ability of the tumor cells to develop resistance (14,15) to growth inhibition by estrogen withdrawal (53), antiestrogen administration (54), and retinoic acid (55). It will be very important to determine whether growth inhibition by TRF is also subject to development of resistance or not. Tocotrienols could offer an alternative clinical approach to growth suppression of breast cancer cells resistant to other regimes of therapy.

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REFERENCES

1. Sebrell, W.H., and Harris, R.S. (1972) *The Vitamins,* 2nd edn., Vol. 5, pp. 172–173, Academic Press, New York.
2. Tappel, A.L. (1972) Vitamin E and Free Radical Peroxidation of Lipids, *Ann. N.Y. Acad. Sci. 203,* 12.
3. Burton, G.W., and Ingold, K.U. (1989) Vitamin E as *in vitro* and *in vivo* Antioxidant, *Ann. N.Y. Acad. Sci. 570,* 7–22.
4. Goh, S.H., Hew, N.F., Ong, A.S.H., Choo, Y.M., and Brumby, S. (1990) Tocotrienols from Palm Oil: Electron Spin Resonance Spectra of Tocotrienoxyl Radicals, *J. Am. Oil Chem. Soc. 67,* 250–254.
5. Serbinova, E., Kagan, V., Han, D., and Packer, L. (1991) Free Radical Recycling and Intermembrane Mobility in the Antioxidation Properties of Alpha-Tocopherol and Alpha-Tocotrienol, *Free Radicals Biol. Med. 10,* 263–275.
6. Ong, A.S.H. (1993) Natural Sources of Tocotrienols in Vitamin E in Health and Disease (Packer, L., and Fuchs, J., eds.) Marcel Dekker, New York, pp. 3–8.
7. Gould, M.N., Haag, J.D., Kennan, W.S., Tanner, MA., and Elson, C.E. (1991) A Comparison of Tocopherol and Tocotrienol for the Chemoprevention of Chemically Induced Rat Mammary Tumors, *Am. J. Clin. Nutr. 53,* 1068s–1070s.
8. Tan, B. (1992) Antitumor Effects of Palm Carotenes and Tocotrienols in HRS/J Hairless Female Mice, *Nutr. Res. 12,* S163–173.
9. Sylvester, P.W., Russell, M., Ip, M.M., and Ip, C. (1986) Comparative Effects of Different Animal and Vegetable Fats Before and During Carcinogen Administration on Mammary Tumourigenesis, Sexual Maturation and Endocrine Function in Rats, *Cancer Res. 46,* 757–762.
10. Sundram, K., Khor, H.T., Ong, A.S.H., and Pathmanathan, R. (1989) Effect of Dietary Palm Oils on Mammary Carcinogenesis in Female Rats Induced by 7,12 Dimethylbenz(α)anthracene, *Cancer Res. 49,* 1447–1451.
11. Kritchevsky, D., Weber, M.M., and Klurfeld, D.M. (1992) Influence of Different Fats (Soyabean Oil, Palm Olein or Hydrogenated Oil) on Chemically-Induced Mammary Tumors in Rats, *Nutr. Res. 12,* S175–179.
12. Nesaretnam, K., Khor, H.T., Ganeson, J., Chong, Y.H., Sundram, K., and Gapor, A. (1992) The Effect of Vitamin E Tocotrienols from Palm Oil on Chemically Induced Mammary Carcinogenesis in Female Rats, *Nutr. Res. 12,* 879–892.
13. Nesaretnam, K., Guthrie, N., Chambers, A.F., and Carroll, K.K. (1995) Effect of Tocotrienols on the Growth of a Human Breast Cancer Cell Line in Culture, *Lipids 30,* 1139–1143.
14. Beck, J.S. (ed.) (1989) Oestrogen and the Human Breast, *Proc. Roy. Soc. Edin. 95B,* 1–307.
15. Stewart, H.J., Anderson, T.J., and Forrest, A.P.M. (eds.) (1991) Breast Cancer: New Approaches, *Brit. Med. Bull. 47,* 251–518.
16. Darbre, P.D., and Daly, R.J. (1989) Effects of Oestrogen on Human Breast Cancer Cells in Culture, *Proc. Roy. Soc. Edin. 95B,* 119–132.
17. Lippman, M.E., and Dickson, R.B. (1990) *Regulatory Mechanisms in Breast Cancer: Advances in Cellular and Molecular Biology of Breast Cancer,* Kluwer Academic Publishers, Boston, pp. 1–452.
18. Engel, L.W., Young, N.A., Tralka, T.S., Lippman, M.E., O'Brien, S.J., and Joyce, M.J. (1978) Establishment and Characterisation of Three New Continuous Cell Lines Derived from Human Breast Carcinomas, *Cancer Res. 38,* 3352–3364.
19. Osborne, C.K., Hobbs, K., and Trent, J.M. (1987) Biological Differences Among MCF-7 Human Breast Cancer Cell Lines from Different Laboratories, *Breast Cancer Res. Treat. 9,* 111–121.
20. Yee, D. (1992) Insulin-Like Growth Factors in Breast Cancer, *Breast Cancer Res. Treat. 22,* 1–106.
21. Kelley, K.M., Oh, Y., Gargosky, S.E., Gucev, Z., Matsumoto, T., Hwa, V., Ng, L., Simpson, D.M., and Rosenfeld, R.G. (1996) Insulin-Like Growth Factor Binding Proteins (IGFBPs) and Their Regulatory Dynamics, *Int. J. Biochem. Cell Biol. 28,* 619–637.
22. Yee, D., Cullen, K.J., Paik, S., Perdue, J.F., Hampton, B., Schwartz, A., Lippman, M.E., and Rosen, N. (1988) Insulin-Like Growth Factor II mRNA Expression in Human Breast Cancer, *Cancer Res. 48,* 6691–6696.
23. Stewart, A.J., Johnson, M.D., May, F.E.B., and Westley, B.R.

(1990) Role of Insulin-Like Growth Factors and the Type I Insulin-Like Growth Factor Receptor in the Estrogen-Stimulated Proliferation of Human Breast Cancer Cells, *J. Biol. Chem. 265*, 21172–21178.
24. Pratt, S.E., and Pollock, M.N. (1993) Estrogen and Antioestrogen Modulation of MCF-7 Human Breast Cancer Cell Proliferation Is Associated with Specific Alterations in Accumulation of Insulin-Like Growth Factor Binding Proteins in Conditioned Media, *Cancer Res. 53*, 5193–5198.
25. Parker, M.G. (ed.) (1991) *Nuclear Hormone Receptors: Molecular Mechanisms, Cellular Functions, Clinical Abnormalities*, Academic Press, London, pp. 1–404.
26. Parker, M.G. (ed.) (1993) *Steroid Hormone Action*, IRL Press, Oxford, pp. 1–210.
27. Wakeling, A.E. (1993) The Future of New Pure Antiestrogens in Clinical Breast Cancer, *Breast Cancer Res. Treat. 25*, 1–9.
28. Perachiotti, A., and Darbre, P.D. (1994) Coculture Inserts Possess an Intrinsic Ability to Alter Growth Regulation of Human Breast Cancer Cells, *Exp. Cell Res. 213*, 404–411.
29. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A., and Chambon, P. (1982) Cloning of cDNA Sequences of Hormone-Regulated Genes from the MCF-7 Human Breast Cancer Cell Line, *Nucleic Acids Res. 10*, 7895–7903.
30. Sundram, K., and Gapor, A. (1992) Vitamin E from Palm Oil Its Extraction and Nutritional Properties, *Lipid Technolog.*, Peter J. Barnes, Bridgwater, England, November/December, pp. 137–141.
31. Darbre, P., Yates, J., Curtis, S., and King, R.J.B. (1983) Effect of Estradiol on Human Breast Cancer Cells in Culture, *Cancer Res. 43*, 349–354.
32. Maniatis, T., Fitsch, F.F., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, pp. 1–545.
33. Brown, A.M.C., Jeltsch, J.M., Roberts, M., and Chambon, P. (1984) Activation of pS2 Gene Transcription Is a Primary Response to Estrogen in the Human Breast Cancer Cell Line MCF-7, *Proc. Natl. Acad. Sci. USA 81*, 6344–6348.
34. Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986) Analysis of Serum Insulin-Like Growth Factor Binding Proteins Using Western Blotting: Use of the Method for Titration of the Binding Proteins and Competitive Binding Studies, *Anal. Biochem. 154*, 138–143.
35. Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V., and Lowry, P.J. (1981) Iodination of Proteins, Glycoproteins and Peptides Using a Solid Phase Oxidising Agent 1,3,4,6-Tetrachloro-3,6-diphenylglycouril (Iodogen), *Anal. Biochem. 117*, 136–146.
36. Corcoran, D., Perachiotti, A., and Darbre, P.D. (1996) Increased Autocrine Production of Insulin-Like Growth Factor II (IGF-II) Alters Serum Sensitivity of MCF7 Human Breast Cancer Cell Proliferation, *Cell Prolif. 29*, 479–493.
37. Carroll, K.K., Guthrie, N., Nesaretnam, K., Gapor, A., and Chambers, A.F. (1995) Anti-Cancer Properties of Tocotrienols from Palm Oil, in *Nutrition, Lipids, Health and Disease* (Ong, A.S.H., Niki, E., and Packer, L., eds.) AOCS Press, Champaign, pp. 117–121.
38. Guthrie, N., Chambers, A.F., Gapor, A., and Carroll, K.K. (1995) *In vitro* Inhibition of Proliferation of Receptor-Positive MCF-7 Human Breast Cancer Cells by Palm Oil Tocotrienols, *FASEB J. 9*, A968 (abstract 5735).
39. Guthrie, N., Gapor, A., Chambers, A.F., and Carroll, K.K. (1997) Inhibition of Proliferation of Estrogen Receptor-Negative MDA-MB-435 and -Positive MCF-7 Human Breast Cancer Cells by Palm Oil Tocotrienols and Tamoxifen, Alone and in Combination, *J. Nutr. 127*, 544S–548S.
40. Guthrie, N., Gapor, A., Chambers, A.F., and Carroll, K.K. (1997) Palm Oil Tocotrienols and Plant Flavonoids Act Synergistically to Inhibit Proliferation of Estrogen Receptor-Negative MDA-MB-231 and -Positive MCF-7 Human Breast Cancer Cells in Culture, *Asia Pacific J. Clin. Nutr. 6*, 41–45.
41. Kyprianou, N., English, H.F., Davidson, N.E., and Isaacs, J.T. (1991) Programmed Cell Death During Regression of the MCF-7 Human Breast Cancer Following Estrogen Ablation, *Cancer Res. 51*, 162–166.
42. Tenniswood, M.P., Guenette, R.S., Lakins, J., Mooibroek, M., Wong, P., and Welsch, J.E. (1992) Active Cell Death in Hormone-Dependent Tissues, *Cancer Metastasis Rev. 11*, 197–220.
43. Warri, A.M., Huovinen, R.L., Martikainen, P.M., and Harkonen, P.L. (1993) Apoptosis in Toremifene-Induced Growth Inhibition of Human Breast Cancer Cells *in vivo* and *in vitro*, *J. Natl. Cancer Inst. 85*, 1412–1418.
44. Pagliacci, M.C., Tognellini, R., Grignani, F., and Nicoletti, I. (1991) Inhibition of Human Breast Cancer Cell (MCF-7) Growth *in vitro* by Somatostatin Analog SMS 201-995: Effects on Cell Cycle Parameters and Apoptotic Cell Death, *Endocrinology 129*, 2555–2562.
45. Armstrong, D.K., Isaacs, J.T., Ottaviano, Y.L., and Davidson, N.E. (1992) Programmed Cell Death in an Estrogen-Independent Human Breast Cancer Cell Line MDA-MB-468, *Cancer Res. 52*, 3418–3424.
46. McCloskey, D.E., Casero, R.A., Woster, P.M., and Davidson, N.E. (1995) Induction of Programmed Cell Death in Human Breast Cancer Cells by an Unsymmetrically Alkylated Polyamine Analogue, *Cancer Res. 55*, 3233–3236.
47. Chatelain, E., Boscoboinik, D.O., Bartoli, G.M., Kagan, V.E., Gey, F.K., Packer, L., and Azzi, A. (1993) Inhibition of Smooth Muscle Cell Proliferation and Protein Kinase C Activity by Tocopherols and Tocotrienols, *Biochim. Biophys. Acta 1176*, 83–89.
48. Gundimeda, U., Chen, Z.H., and Gopalakrishna, R. (1996) Tamoxifen Modulates Protein Kinase C *via* Oxidative Stress in Estrogen Receptor-Negative Breast Cancer Cells, *J. Biol. Chem. 271*, 13504–13514.
49. Koba, K., Abe, K., Ikeda, I., and Sugano, M. (1992) Effects of Alpha-Tocopherol and Tocotrienols on Blood Pressure and Linoleic Acid Metabolism in the Spontaneously Hypertensive Rat, *Biosci. Biotechnol. Biochem. 56*, 1420–1423.
50. Rose, D.P., Connolly, J.M., and Liu, X.H. (1994) Effects of Linoleic Acid on the Growth and Metastasis of Two Human Breast Cancer Cell Lines in Nude Mice and the Invasive Capacity of These Cell Lines *in vitro*, *Cancer Res. 54*, 6557–6562.
51. Glover, J.F., Irwin, J.T., and Darbre, P.D. (1988) Interaction of Phenol Red with Estrogenic and Antiestrogenic Action on Growth of Human Breast Cancer Cells ZR-75-1 and T-47-D, *Cancer Res. 48*, 3693–3697.
52. Gucev, Z.S., Oh, Y., Kelley, K.M., and Rosenfeld, R.G. (1996) Insulin-Like Growth Factor Binding Protein 3 Mediates Retinoic Acid- and Transforming Growth Factor β2-Induced Growth Inhibition in Human Breast Cancer Cells, *Cancer Res. 56*, 1545–1550.
53. Darbre, P.D., and Daly, R.J. (1990) Transition of Human Breast Cancer Cells from an Oestrogen Responsive to Unresponsive State, *J. Steroid Biochem. Molec. Biol. 37*, 753–763.
54. Horwitz, K.B. (1994) How Do Breast Cancers Become Hormone Resistant? *J. Steroid Biochem. Molec. Biol. 49*, 295–302.
55. Stephen, R., Corcoran, D., and Darbre, P.D. (1996) Retinoic Acid Inhibits Growth of Breast Cancer Cells in the Short-Term but Not the Long-Term, *Biochem. Soc. Trans. 24*, 365S.

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