Tocotrienol-Rich Fraction from Palm Oil Affects Gene Expression in Tumors Resulting from MCF-7 Cell Inoculation in Athymic Mice

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ABSTRACT: It has recently been shown that tocotrienols are the components of vitamin E responsible for inhibiting the growth of human breast cancer cells *in vitro*, through an estrogen-independent mechanism. Although tocotrienols act on cell proliferation in a dose-dependent manner and can induce programmed cell death, no specific gene regulation has yet been identified. To investigate the molecular basis of the effect of tocotrienols, we injected MCF-7 breast cancer cells into athymic nude mice. Mice were fed orally with 1 mg/d of tocotrienol-rich fraction (TRF) for 20 wk. At end of the 20 wk, there was a significant delay in the onset, incidence, and size of the tumors in nude mice supplemented with TRF compared with the controls. At autopsy, the tumor tissue was excised and analyzed for gene expression by means of a cDNA array technique. Thirty out of 1176 genes were significantly affected. Ten genes were down-regulated and 20 genes up-regulated with respect to untreated animals, and some genes in particular were involved in regulating the immune system and its function. The expression of the interferon-inducible transmembrane protein-1 gene was significantly up-regulated in tumors excised from TRF-treated animals compared with control mice. Within the group of genes related to the immune system, we also found that the CD59 glycoprotein precursor gene was up-regulated. Among the functional class of intracellular transducers/effectors/modulators, the c-myc gene was significantly down-regulated in tumors by TRF treatment. Our observations indicate that TRF supplementation significantly and specifically affects MCF-7 cell response after tumor formation *in vivo* and therefore the host immune function. The observed effect on gene expression is possibly exerted independently from the antioxidant activity typical of this family of molecules.

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Vitamin E is a generic term referring to an entire class of compounds that is further divided into two subgroups called tocopherols and tocotrienols. Just as there are several forms of tocopherols (α, β, γ, and δ), there are also α-, β-, γ-, and δ-tocotrienols. Although tocopherols are found abundantly in oils extracted from soybean, olive, cottonseed, and sunflower seed, tocotrienols are found only in the oil fractions of some cereal grains such as wheat, barley, rice, and, most abundantly, the fruit of palm (1). Commercial quantities of tocotrienols are, in fact, extracted from palm oil and rice bran oil. A standardized tocotrienol-rich fraction (TRF) composed of 32% α-tocopherol and 68% tocotrienols can be obtained from palm oil after esterification and following distillation, crystallization, and chromatography (2).

Recent work has shown that tocotrienols, but not tocopherols, can exert direct inhibitory effects on cell growth in human breast cancer cell lines *in vitro* (3–5) and also induce cells to undergo apoptosis (6). The inhibitory effect, however, occurs irrespective of the estrogen receptor status of the cells (7). The inhibitory effect on cell growth is more pronounced with γ- and δ-tocotrienols (5,7). The mechanism of action is not yet completely understood, with previous data suggesting that the action is independent from an antagonism with estrogen activity (7). However, it has been reported that α-tocopherol itself has no inhibitory activity on breast cancer cell growth (3,4,7,8). Tocotrienols are also reported to have a pro-apoptotic effect on several lines of tumor cells (6,9,10). McIntyre and coworkers (10) have also shown that highly malignant cells are more sensitive to the antiproliferative and apoptotic effects of tocotrienols in comparison with preneoplastic cells.

Although vitamin E (both tocopherols and tocotrienols) is a potent antioxidant, the antitumor activity of vitamin E may not be associated with its antioxidant activity (11). It is thought that vitamin E exerts its antitumor activity by modulating a number of intracellular signaling pathways involved in mitogenesis (12–14) and apoptosis (10,12,15). Oral administration of tocopherols and tocotrienols also reportedly affects the immune system and the proliferation of spleen and mesenteric lymph node lymphocytes (16). We have preliminary evidence suggesting that the number of natural killer (NK) cells increases in nude mice supplemented with tocotrienols and challenged with MCF-7 human breast cancer cells (Nesaretnam, K., unpublished data). Other researchers also have reported that oral administration of tocopherols and

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Abbreviations: APC, antigen-presenting cell; FcRn, IgG Fe receptor large subunit P51 precursor; IFITM-1, interferon-inducible transmembrane protein-1; IFN-γ, interferon-γ; MHC, major histocompatibility; NK, natural killer; TRF, tocotrienol-rich fraction.

tocotrienols can affect the immune system and the proliferation of spleen and mesenteric lymph node lymphocytes (16). However, the mechanism by which vitamin E helps to boost the immune system to fight tumor growth and spread is not known.

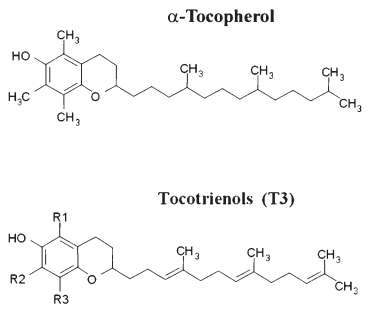
The *in vivo* study reported herein was designed on the basis of a heterogeneic model (human breast cancer cells implanted into a rodent host) in order to (i) specifically focus on the effect of TRF on the growth of cancer cells rather than on the host, and (ii) assess whether implantation into the host results in a different pattern of gene expression and sensitivity to tocotrienols in the tumor mass. Even though the second issue might appear quite obvious, no current studies are able to provide a reliable database to either support or discard this hypothesis.

To investigate the molecular basis of the effect of tocotrienols on tumor growth, we administered a standardized mixture of tocotrienols extracted from palm oil and performed an array analysis of cancer-related gene expression on tumors resulting from the inoculation of MCF-7 cells in control and TRF-supplemented athymic nude mice.

MATERIALS AND METHODS

Isolation of the TRF. The TRF was obtained from Golden Hope Plantation (Ipoh, Malaysia). TRF has a standardized composition of 32% α-tocopherol, 25% α-tocotrienol, 29% γ-tocotrienol, and 14% δ-tocotrienol. The chemical structure of tocotrienols present in TRF is shown in Figure 1. Extraction of TRF from palm oil has been described by Sundram and Gapor (2). Briefly, 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%.

Analysis of tocotrienols and tocopherols. The tocopherols and tocotrienols were analyzed by HPLC. The system used was a Shimadzu LC-10AT high-performance liquid chromatograph coupled with a Shimadzu RF-10AXL fluorescence spectrophotometer, Shimadzu Class VP data acquisition software, and a silica column (YMC A-012, 150 × 6 mm i.d., 5 μm; YMC Co., Ltd., Kyoto, Japan). The eluting solvent was hexane/isopropyl alcohol (99.5:0.5 vol/vol) at a flow rate of 2.0 mL/min. The detector was set at an excitation wavelength of 295 nm and an emission wavelength of 325 nm. A 500-mg sample of tissue was homogenized with a 4:1:1 mixture of hexane/ethanol and 0.9% sodium chloride at 11,000 rpm for 5 min. The homogenate was then centrifuged at 800 × *g* for 5 min. The resulting supernatant was filtered and evaporated using a rotary evaporator. A known amount of lipid sample was dissolved in a 10-mL volumetric flask using the eluting solvent, and 10 μL of the solution was injected into the HPLC system. A standard solution of a mixture of α-tocopherol and α-, γ-, and δ-tocotrienols was also injected using the same procedure. Quantification of the major components of vitamin E was carried out by comparing the peak areas of the components with those of the standards.



	R1	R2	R3
α-T3	CH_3	CH_3	CH_3
γ-T3	H	CH_3	CH_3
δ-T3	H	H	CH_3

FIG. 1. Structure of vitamin E (tocopherol and tocotrienol) components in the tocotrienol-rich fraction (TRF) from palm oil.

Culture of stock cells. MCF-7 human breast cancer cells were kindly provided by Dr. Kent C. Osborne at passage number 390 (17). Stock cells were grown as monolayer cultures in DMEM supplemented with 5% FCS (Gibco, BRL Life Technologies Incorporated, Grand Island, NY) and 10^{-8} M estradiol in a humidified atmosphere of 5% carbon dioxide in 95% air at 37°C. Cells were subcultured at weekly intervals by suspension with 0.06% trypsin/0.02% EDTA (pH 7.3). Tumor cells were harvested for inoculation by incubating briefly with 0.06% trypsin and 0.02% EDTA. The tissue culture flask was tapped to dislodge the cells, which were then resuspended in DMEM supplemented with 5% FCS. Tumor cells intended for inoculation were washed by centrifugation and resuspended in DMEM.

Animals. Forty female athymic nude mice (NCR nu/nu), 3 to 4 wk old, were obtained from the Animal Breeding Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. The animals were housed under specific pathogen-free conditions in micro-isolator cages. The care and treatment of the experimental animals conformed to the guidelines of the Institute for Medical Research for the ethical treatment of laboratory animals. Animals were randomly divided into an experimental and a control group. Both the control and the experimental groups were treated topically with 10^{-5} M estradiol and fed a commercial mouse pellet diet for 20 wk and then sacrificed before excising the tumor mass. The experimental group was supplemented with 1 mg/d of TRF (dissolved in palm oil) by gavage on the same day as tumor cell inoculation (see next paragraph), whereas control animals were treated with the carrier only.

Tumor inoculation. Animals were anesthetized with phentobarbital, and tumor cells injected into a right-sided thoracic mammary fat pad that had been exposed by a small incision (5 mm). The mammary fat pad was exposed, and a 50-μL volume of inoculum containing 10^6 cells was injected into the tissue through a 27-gauge needle. By exposing the fat pad, we were able to ensure that the cells were injected into the tissue

and not into the subcutaneous space. The incision was then closed with a skin clip. Mice were weighed, and the inoculation site was palpated at weekly intervals by an investigator who was blinded to the treatments in order to evaluate tumor surface area. TRF treatment was not associated with any significant changes in growth rate and final body weight in comparison with the group of control animals (data not shown). The experiment was terminated 20 wk after injection of the tumor cells, and the mice were killed by CO_2 euthanasia. At necropsy, body and tumor weights were determined.

RNA isolation and purification. Total RNA was extracted from pooled tumor tissues from three randomly selected animals using a Trizol solution (BRL Life Technologies) according to the manufacturer's instructions, with some minor modifications, in order to obtain sufficient RNA for the array analysis. Tissues and cells were homogenized in the Trizol solution and incubated for 15 min at room temperature. After the addition of a 20% volume of chloroform, homogenates were vortexed for 2 min and centrifuged at 12,000 × *g* for 20 min at 4°C. The resulting inorganic phase was subjected to three extractions with acid phenol/chloroform/isoamyl alcohol (125:24:1; Ambion, Austin, TX) and one extraction with chloroform. RNA was precipitated overnight at 4°C with 0.75 vol of 7.5 M LiCl and then centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was resuspended in 400 μL of distilled water, reprecipitated with 40 μL of 3 M Na acetate (pH 5.2) and 1 mL of ethanol, and washed with 70% ethanol. RNA integrity was checked by denaturing gel electrophoresis. Before labeling, total RNA was treated with 25 units of DNase I to remove any contaminating DNA.

cDNA labeling and Atlas™ Cancer Array membrane hybridization, exposure, and analysis. Atlas™ Human Cancer cDNA Expression Arrays (Cat. #7851-1) were purchased from Clontech Laboratories Inc. (Palo Alto, CA). Array membranes contained 10 ng of each gene-specific cDNA from 1176 known genes and 9 housekeeping genes (see http://www.clontech.com for the complete list of genes). Poly A^+ RNA enrichment, cDNA Probe Synthesis, and purification were performed using the Atlas Pure RNA Labeling system (Clontech) following the manufacturer's instructions, starting from 50 μg total RNA and using [α-^{32}P]dATP (NEN, Boston, MA). Membrane arrays were hybridized for 18 h at 68°C into rolling bottles with 5 mL ExpressHyb hybridization solution (Clontech) containing the denatured probes (10×10^6 cpm) and 5 μg Cot-1 DNA. The membranes were then washed in bottles for 2 h at 68°C in 2× SSC, 1% SDS with three changes of solutions, and then for 30 min at 68°C in 0.1× SSC, and 0.5% SDS. Membranes were finally rinsed in 2× SSC at room temperature and exposed to X-ray films (Kodak Biomax from Kodak, Rochester, NY, or Amersham MP from Amersham, Piscataway, NJ) at −70°C for 1 to 6 d. Films were acquired with a scanner for transparencies, and images were analyzed with AtlasImage software (Version 2.01, Clontech). The software analysis results were confirmed by visual inspection of hybridization signals to ensure reliability. Since tocotrienols were found to modulate the expression of one of the housekeeping genes included in the array (see the Results section), a global gene-normalization method was preferred. In such a method, the normalization coefficient is calculated using the average value of all genes in the array instead of using only the housekeeping genes.

Reproducibility and precision limits. Previous studies addressing the application of a cDNA array have indicated that the CV for differential gene expression in cultured cells is 10–15% (18). Studies on the reproducibility and variability of array results have indicated that a difference of twofold or greater in the expression of a particular gene is to be considered a real difference in transcript abundance (19,20). Data are reported according to the suggested standardization of microarray experiments (minimal information about a microarray experiment) (21). A difference in gene expression between the TRF-treated samples and controls was therefore considered significant at a ratio of twofold or more and when both readings had a signal intensity above 1000 units. Data discussed herein were confirmed by a Northern blot hybridization technique (see next paragraph).

Northern blot hybridization. Ten micrograms of total RNA separated through electrophoresis in 1.2% agarose gels was blotted onto Genescreen-N nylon membranes (DuPont, Wilmington, DE) and hybridized according to the manufacturer's instructions. Gene transcripts of MIC-1, CD74/Ii, and the interferon-inducible transmembrane protein-1 (IFITM-1) were detected using [α-^{32}P]dATP (NEN) random primed DNA ampliclones (Boehringer Ingelheim, Ingelheim, Germany) obtained by PCR using sequence-specific primers (see Table 1 for sequences). Normalization of gene expression was achieved by hybridization of the same membranes with a labeled PCR fragment of the GAPDH gene.

Statistical analysis and data presentation. Statistical analysis was performed using the SPSS program (SPSS Inc., Chicago, IL). Fisher's exact test was used to test for a significant difference in the incidence and size of tumors between experimental and control mice. Scion Image® software was used for quantification of the transcripts' relative abundance in Northern blot experiments. The figures present one out of at least three separate experiments providing similar results.

TABLE 1
Nucleotide Sequences (from 5′ to 3′) of Primers Used for the Preparation of Probes in Northern Blot Hybridizations

Genes	Forward	Reverse
MIC-1	CGC GCA ACG GGG ACC ACT	TGA GCA CCA TGG GAT TGT AGC
CD74/Ii	ACC TCA TCC CAT GAG ACC TG	TCC AAA ACA TTG GCT CTT CC
IFITM-1	TGC ACA AGG AGG AAC ATG AG	TGA ATC CAA TGG TCA TGA GG
GAPDH	TGA AGG TCG GAG TCA ACG GAT TTG G	CAT GTG GGC CAT GAG GTC CAC CAC

TABLE 2
Tocopherol and Tocotrienol Concentrations in Plasma, Liver, and Adipose Tissue in Control Animals and in Animals Fed 1 mg/d of a Tocotrienol-Rich Fraction (TRF) by Gavage[a]

Tissue	Total tocopherols	Total tocotrienols
Plasma		
Control	1.88 ± 0.34	1.03 ± 0.66
TRF treatment	3.89 ± .64	6.72 ± 1.79
Liver		
Control	2.36 ± 0.89	0.30 ± 0.06
TRF treatment	7.73 ± 3.90	6.35 ± 4.79
Adipose tissue		
Control	7.46 ± 3.12	1.57 ± 0.54
TRF treatment	20.21 ± 6.48	74.16 ± 23.91

[a]Data are presented as the mean ± SD of 10 animals per group and expressed as μg/mL (plasma) and μg/g tissue (liver and adipose tissue).

RESULTS

We previously reported the effect of TRF on MCF-7 cell proliferation *in vitro* (6,7,9,10). In the present study, we found TRF supplementation to be associated with a significant inhibition of tumor growth *in vivo*.

Effect of TRF on plasma and tissue levels of tocopherols and tocotrienols. The administration of TRF resulted in a significant increase in tocopherol and tocotrienol concentrations in the plasma and tissues of animals in comparison with the control group (see Table 2). The levels of tocopherol increased in all tissues by about threefold. As expected, the increase in tocotrienol levels associated with TRF administration was much more evident than that of tocopherol levels: In plasma we observed levels sevenfold higher than in control animals, and an even more dramatic increase was observed in the liver and adipose tissue (more than 20-fold and about 50-fold, respectively), confirming that selected tissues are able to accumulate and store tocotrienols available in the diet (22).

Effect of TRF on tumor development in nude mice after MCF-7 cell inoculation. The cumulative incidence of mammary fat pad tumors in the two groups of animals over a period of 20 wk is shown in Figure 2A. Tumor growth was detected in the control group (i.e., mice not supplemented with TRF) 2 wk after inoculation of the MCF-7 cells. In addition, by week 7 the incidence of mammary fat pad tumors was 100% (i.e., detected in all mice in the control group). On the other hand, TRF-supplemented mice started to develop palpable tumors only from the seventh week of inoculation and, more importantly, only 50% of the mice in this group had tumors at week 14. TRF supplementation also affected tumor size. Tumor surface area was in fact significantly reduced in treated animals, being on the average about one-third that of control animals at the end of the experimental period (see Figure 2B).

Effect of TRF supplementation on gene expression in tumors resulting from inoculating nude mice with MCF-7 cells: cDNA array data. Two hundred and forty-six genes were significantly hybridized, with a total of 1185 cDNA spotted onto the membrane. The expression of 30 genes (i.e., 12% of the total number of hybridized genes) was significantly affected by the supplementation of TRF from palm oil *in vivo* in the tumor mass that resulted after 20 wk of inoculation of MCF-7 into nude mice. Among these 30 genes, 20 genes were up-regulated and 10 were down-regulated (see Table 2). The complete list of genes whose expression was affected following TRF supplementation in the tumor tissue is presented in Table 3. Genes have been classified into functional groups according to the putative function of the encoded proteins. However, the data discussed hereafter focus on only a selected subsample of genes involved in specific groups.

In tumors excised from nude mice, the expression of the CD74/Ii gene displayed the highest up-regulation (11-fold), whereas the IgG Fc receptor large subunit P51 precursor (FcRn) displayed a significant down-regulation (3.3-fold). The expression of the IFITM-1 gene was significantly up-regulated in tumor cells following TRF treatment with respect to control mice. Within the group of genes related to the immune system, we also found that the CD59 glycoprotein precursor gene was also up-regulated by TRF. Among the 30 genes modulated by TRF supplementation in MCF-7-induced tumors, nine genes could be functionally classified as intracellular transducers/effectors/modulators (see Table 2). This category includes the c-myc gene, which is a gene that was significantly down-regulated in tumors after TRF treatment.

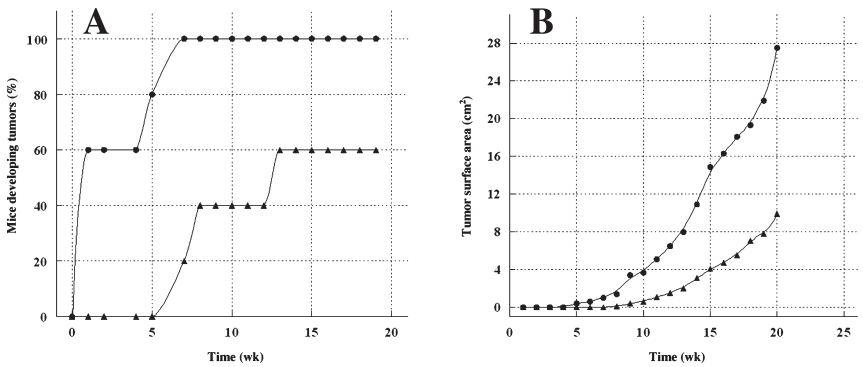


FIG. 2. Cumulative tumor incidence (A) and tumor surface area (B) in control and TRF-supplemented nude mice inoculated with MCF-7 cells. There were 20 animals in each group. -●- = Control animals; -▲- = TRF treatment. For abbreviation see Figure 1.

TABLE 3
Fold Changes in Gene Expression in MCF-7-Induced Tumors in Nude vs. Control Mice[a]

Genes	Gene bank no.	Treated vs. control
Oncogenes and tumor suppressors		
IFITM-1 (interferon-inducible protein 9-27)	J04164	2.2
c-myc oncogene	V00568	0.5
Prohibitin (PHB)	S85655; U17179	0.4
Metalloproteinase inhibitor 3 precursor; tissue inhibitor of metalloproteinases 3 (TIMP3); mitogen-inducible gene 5 (MIG5)	Z30183	5.1
Synapse-associated protein 102 (SAP102); neuroendocrine-DLG [NE-DLG; DLG3; human homolog of Drosophila discs large (DLG)]	U49089	3.1
p33ING1	AF001954	3.5
Apoptosis-associated proteins and DNA-binding and -damage signaling/repair proteins		
BCL-2 binding athanogene-1 (BAG-1); glucocorticoid receptor-associated protein (RAP46)	S83171; Z35491	2.4
Induced myeloid leukemia cell differentiation protein (MCL-1)	L08246	2.4
Fas-activated serine/threonine (FAST) kinase	X86779	2.2
DNA-repair protein XRCC1	M36089	0.5
Immune system proteins		
IgG receptor FC large subunit P51 precursor (FCRN); neonatal FC receptor; IgG FC fragment receptor transporter alpha chain	U12255	0.3
HLA-DR antigen-associated invariant subunit (CD74/Ii ANTIGEN)	X00497	11.5
CD59 glycoprotein precursor; membrane attack complex inhibition factor (MACIF); MACIP; MEM43; MIRL; HRF20; 1F5	M34671	2.9
Extracellular cell-signaling and cell receptors		
Macrophage inhibitory cytokine 1 (MIC1)	AF019770	2.9
Intracellular transducers/effectors/modulators		
Serine/threonine-protein kinase PCTAIRE 1 (PCTK1)	X66363	0.4
Placental calcium-binding protein; calvasculin; S100 calcium-binding protein A4; MTS1 protein	M80563	2.4
Zyxin + zyxin-2	X94991; X95735	2.1
PTPCAAX1 nuclear tyrosine phosphatase (PRL-1)	U48296	2.1
Transcription activators/repressors		
TAX1-binding protein 151 (TXBP151)	U33821	2.6
Transcription factor erf-1; AP2 gamma transcription factor	U85658	2.5
Metabolism		
Methylenetetrahydrofolate dehydrogenase-methylenetetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase	J04031	0.4
Purine nucleoside phosphorylase (PNP); inosine phosphorylase	X00737	0.4
L-Lactate dehydrogenase M subunit (LDHA)	X02152	0.4
Cell adhesion proteins		
40S ribosomal protein SA (RPSA); 34/67-kDa laminin receptor (LAMR1); colon carcinoma laminin-binding protein; NEM/1CHD4	U43901	0.4
Integrin alpha 3 (ITGA3); galactoprotein B3 (GAPB3); VLA3 alpha subunit; CD49C antigen	M59911	5.2
Membrane channels and transporters		
PKU-alpha	AB004884	2.2
Trafficking and extracellular matrix proteins		
Fibronectin precursor (FN)	X02761; K00799; K02273; X00307; X00739	3.6
TRAM protein	X63679	2.2
Type I cytoskeletal 10 keratin; cytokeratin 10 (K10)	M19156	0.4
Type I cytoskeletal 14 keratin; cytokeratin 14 (K14; CK14)	J00124	3.5

[a]Changes greater than twofold or less than 0.5-fold were considered significant and included in the table.

Northern blot hybridization. RNA extracted from tumors that grew in TRF-supplemented mice and control mice was subjected to Northern blot hybridization to confirm data obtained by means of cDNA array analysis. We performed Northern blot analyses on only a selected number of genes, i.e., MIC-1, CD74, IFITM-1, and GAPDH. Their hybridization signals, together with the quantification obtained after normalization to the expression of GAPDH, are shown in Figure 3. Northern blotting confirmed the up-regulation of MIC-1 (about tenfold), CD74/Ii (about fourfold), and IFITM-1 (about twofold) gene expression in MCF-7-induced tumors in nude mice fed TRF in comparison with control animals.

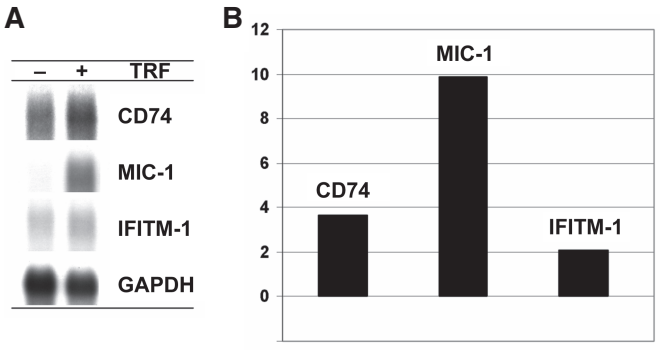


FIG. 3. (A) Northern blot analyses of TRF effects on the expression of MIC-1, CD74, and IFITM-1 genes in the tumor mass resulting from MCF-7 inoculation in nude mice. (B) Fold changes in gene expression calculated using Scion software and normalized by hybridization signals of the GAPDH gene. For abbreviation see Figure 1.

DISCUSSION

We have previously reported that TRF inhibits proliferation of estrogen receptor-positive and estrogen receptor-negative human breast tumor cell lines *in vitro* (4,7,23). Moreover, several studies have shown that high dietary intake of tocotrienols can suppress carcinogen-induced mammary tumorigenesis in experimental animals (24,25).

This study reports the effect of the administration of TRF, a standardized tocotrienol-rich extract from palm oil, on tumor incidence resulting from inoculation of MCF-7 breast cancer cells into athymic nude mice. Our data show that at the end of the experimental period of 20 wk, there was a significant delay in the onset, incidence, and size of tumor growth in nude mice supplemented with TRF compared with the controls, which did not receive any TRF. As expected, TRF supplementation was associated with a significant increase in both tocopherol and tocotrienol concentrations in plasma, liver, and adipose tissue, the concentration of tocotrienols being much more dramatic than that of tocopherols, particularly in the adipose tissue (about 50-fold with respect to the unsupplemented animals).

Differential gene expression in the tumor mass was assessed by means of a macroarray analysis, which allowed the identification of a number of genes modulated by the supplementation of TRF in the tumor mass. In this paper, we included a complete list of genes affected by TRF treatment in tumors excised from TRF-treated and -untreated animals. However, we focus our discussion herein on some of the genes involved in the regulation of the immune system and its function. In fact, some preliminary data suggest that TRF from palm oil is able to exert an immunomodulatory role in nude mice (unpublished data), possibly due at least in part to a response to signals secreted from tumor cells. In accordance with this hypothesis, lines of evidence indicate that the likelihood of tumor growth is a consequence of a balance between the host immune response and tumor growth kinetics. The initiation of a host immune response toward the tumor is dependent on the activation of T-lymphocytes that can recognize tumor cells as abnormal. The activation of naïve T-lymphocytes requires two distinct signals (26,27). The first one is delivered following the engagement of the T-cell receptor with the major histocompatibility (MHC)-peptide complex on an antigen-presenting cell (APC). The second signal, which is a costimulatory signal (28), is delivered following the binding of coreceptors on the T-cell with its respective ligands on the APC. Although tumors may be able to deliver antigen-specific signals to T-cells, they are generally unable to deliver the necessary costimulatory signals required for the full activation of naïve T-lymphocytes (26,29,30). Antigen recognition in the absence of the second signal will render T-cells nonreactive or anergic (31), or it may cause these cells to be deleted from the host T-cell repertoire.

Recent evidence (32) indicates that peripheral solid tumors usually evade the host immune response. Hence, one of the therapeutic approaches to curbing the growth and spread of tumors would be to find a more effective way of inducing a cell-mediated immune response where both NK cells and cytotoxic T-lymphocytes can be appropriately activated (33).

The MHC-class Ii-associated invariant chain (CD74/Ii) reportedly plays a central role in the biological function of the MHC class Ii molecules (34). The invariant chain plays a critical role in the presentation of processed peptides to the $CD4^+$ T-lymphocytes by influencing the expression and peptide loading of the MHC class II molecules (35). We have observed that TRF supplementation causes the expression of the CD74/Ii gene to be strongly up-regulated in MCF-7-induced tumors of animals fed TRF (see Fig. 3). This observation suggests that the antitumor effect of tocotrienols may be the result of increasing the host immune functions. Several indications support this hypothesis. It has been reported that the treatment of normal human monocytes and macrophages with interferon-γ (IFN-γ) markedly enhances the expression of the CD74/Ii protein in these cells (36). Moreover, Balkwill and collaborators (37) showed that the expression of the CD74/Ii gene on solid tumors of nude mice can be modulated in a dose-dependent fashion and reversibly by IFN-γ administration. Such regulation involves a signal transduction pathway activated in lymphoma and normal mouse B cells by protein kinase C and in controlling the stability of CD74/Ii messenger RNA (38). Therefore, the antineoplastic activity of TRF could be mimicking the effects of an IFN-γ treatment, which in turn can cause an increased expression of the CD74/Ii gene by macrophages and T-lymphocytes.

In agreement with the expression of CD74/Ii, we also observed the up-regulation of the interferon-inducible protein-1 (IFITM-1, IFI17, or 9-27) in MCF-7-induced tumors. Gutterman (39) suggested that expression of this membrane protein, which was identified for its inducibility by interferons, is necessary for the antiproliferative effect of interferons. IFITM-1 has been shown to be a component of a multimeric complex involved in the transduction of antiproliferative and adhesion signals (40). The up-regulation of this gene in tumors from tocotrienol-treated nude mice injected with MCF-7 parallels the up-regulation of CD74/Ii and reinforces the idea of

tocotrienols being able to mimic an interferon-like activation of immune functions through the IFITM-1 and CD74/Ii molecules.

Interferons are also known to exert their inhibitory effect on cell growth by acting at many levels, such as directly affecting the function of proteins (c-myc and Rb) involved in the cell cycle (39). In our array analysis, we detected a significant down-regulation of c-myc gene expression in tocotrienol-treated MCF-7-induced tumors (see Table 2). The c-myc down-regulation by tocotrienols is in agreement with the *in vivo* inhibition in growth by tocotrienols and tocopherols reported by Gu and coworkers (16). This is a very promising finding as the expression of the c-myc oncogene has been implicated in malignant progression in a variety of human tumors (41,42).

Both the array analysis and Northern blot hybridization indicated that TRF up-regulated the expression of MIC-1, a member of the transforming growth factor-β superfamily (43) in tumors resulting from MCF-7 cell injection (see Fig. 2). Although MIC-1 is not expressed in monocytes/macrophages or undifferentiated resting cells, it is progressively up-regulated upon differentiation of these cells by a number of activation agents (44). It has been suggested that MIC-1 could be an autocrine inhibitor of macrophage activation (45,46). Albertoni and collaborators (47), reporting on the antitumorigenic properties of MIC-1 in nude mice, identified this cytokine as a prominent target gene of p53 in the glioblastoma cell line and as a mediator of cellular stress signaling. Moreover, large amounts of MIC-1 are also present in the amniotic fluids and placental extracts, increasing in the sera of pregnant women. It has been suggested that MIC-1 may promote fetal survival by suppressing the production of maternally derived proinflammatory cytokines within the uterus (48,49). Our finding suggests that one of the mechanisms by which tumor cells can escape host immune surveillance may involve the expression of the MIC-1 gene. The role of MIC-1 in inflammatory processes and as a possible means of escape from host immune surveillance requires more investigation.

cDNA array analyses identified two other TRF-sensitive genes coding for immune system proteins: the FcRn (neonatal Fc receptor) and the glycoprotein CD59. FcRn is an MHC complex class I-related receptor that plays a role both in the passive delivery of immunoglobulin from the mother to the fetus during colostrum formation and in the regulation of serum IgG transport to tissues (50). CD59 is a potent complementary inhibitor protein. Durrant and Spendlove (51) reported that CD59 binding to the membrane is able to inhibit the formation of the membrane attack complex (MAC) on the surface of tumor cells, thus inhibiting the direct cytolytic activity of the MAC against tumor cells. Even though more studies are warranted to understand the role of these immune-related genes, TRF appears to have the potential to modulate host immune function in our breast cancer model.

Our study suggests that TRF from palm oil is able to support the host in fighting tumor growth and spread, possibly by modulating the immune response. Even though TRF also provides a significant proportion of tocopherols, previous studies carried out *in vitro* have indicated that tocotrienols, but not tocopherols, significantly affect breast cancer cell growth (3,4). In a recent study (52) conducted on nude mice supplemented with tocopherol succinate, the authors showed reduced tumor incidence when tocopherol succinate was given intraperitoneally. However, when tocopherol succinate was given orally, the effect was lost. The authors speculate that the oral administration failed because, once ingested, vitamin E succinate was hydrolyzed to vitamin E.

These reports suggest that the effect on gene expression we observed in the present study is to be ascribed to tocotrienols. This could be due to the induction of protein expression involved in cell growth inhibition, such as IFITM-1. More studies are needed to test the possibility that tocotrienols are able to induce the production of interferon in the host. Tocotrienols may also exert their antitumor effects by inhibiting the ability of the breast tumor cells to escape from the host immune system by inhibiting the expression of CD74/Ii in tumor cells.

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