Abstract. Background: Tocotrienols, a subgroup of the vitamin E family, have demonstrated antioxidant and anticancer properties. Differential growth responses among different types of tocotrienols have been observed in breast cancer cells; however, specific bioactivity of each individual tocotrienol remains to be elucidated. Materials and Methods: In this study, the effects of γ-tocotrienol were examined with regard to its ability to suppress cell proliferation via modulation of cell cycle regulatory protein expression, and also from the perspective of control of cellular oxidoreductive status through regulation of detoxification enzymes, e.g., quinone reductase NQO2, using estrogen receptor-positive MCF-7 human breast cancer cells. Results: It was shown that treatment by γ-tocotrienol suppressed MCF-7 cell proliferation in a dose- and time-dependent manner. Growth suppression by γ-tocotrienol was accompanied by changes in the levels of cell cycle regulatory proteins, notably, Rb/E2F complex, cyclin D1/cdk4 and cyclin B1/cdk1, as exemplified by loss of cyclin D1, inhibition of specific Rb phosphorylation (pRb-p at Thr821), and by the time- and dose-dependent increase in the expression of NQO2. Conclusion: By exerting control on expression of specific cell cycle regulatory proteins in concomitance with suppression of cell proliferation, as well as the induction of NQO2, γ-tocotrienol offers promise as an added chemopreventive and/or chemotherapeutic agent against breast cancer carcinogenesis.

Breast cancer (BCa) is the most common cause of cancer among women worldwide. In the U.S.A., there is a 1 in 8 chance that women will develop BCa during their lifetime and a 1 in 33 chance that death will result from BCa. This form of neoplastic disease remains a formidable health challenge to women in the U.S.A. (1); therefore, the need for the development of preventive strategies and effective therapies to reduce BCa risk and mortality is imperative. Chemoprevention is among the cadre of approaches introduced for cancer management in recent years. Of note, a wealth of evidence has been accumulated based on case-control, cohort and ecological studies demonstrating that frequency of consumption of fruits and vegetables is positively correlated with increased survival of recurrent BCa cases and reduced death risks (2, 3). The discovery and identification of naturally occurring compounds with chemopreventive attributes are considered mechanistically and clinically important for BCa prevention.

Vitamin E occurs in nature and can be subdivided into structurally related tocopherols containing a fully saturated side-chain and tocotrienols containing an unsaturated side-chain with three double bonds. Both tocopherols and tocotrienols are potent antioxidants, but only tocotrienols display significant anticancer activity in BCa and other tumor cells (4-7). Differential growth responses among different types of tocotrienols were observed in both estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-231 human BCa cells (4, 8-12). γ-Tocotrienol has been reported as an effective growth inhibitor against human BCa (8); however, the mechanistic link between BCa carcinogenesis and γ-tocotrienol remains incompletely understood. In this study, MCF-7 cells were used to further examine the role of proteins involved in controlling cell cycle phase transition as correlates with γ-tocotrienol-elicited suppression of cell proliferation. Furthermore, the relationship between γ-tocotrienol and control of phase II detoxification enzyme NQO2 expression was also assessed. The choice of NQO2 stems from studies showing that the attenuation of cancer development and reduction of cancer mortality relate to up-regulation of phase II defense genes (13), and importantly, is also based on recent evidence showing that NQO2, a phase II detoxification enzyme, is an effective catalyst for the reduction and detoxification of potentially reactive estrogen quinones, considered risk factors for BCa (14).

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Materials and Methods

Reagents. γ-Tocotrienol was obtained from Cayman Chemicals (Ann Arbor, MI, USA). The primary antibodies anti-Rb, anti-E2F, anti-cyclin D1, anti-cyclin B1, anti-cdk4, anti-cdk1, anti-NQO2 and anti-actin, and the secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pRb Ser780, anti-pRb Ser807/811, and anti-phb Thr821 were obtained from BioSource International, Inc. (Camarillo, CA, USA). Fetal bovine serum (FBS); MEM (Minimum Essential Medium Eagle) medium, penicillin and streptomycin were obtained from Cellgro Inc. (Herndon, VA, USA). All the other chemicals and solvents used were of analytical grade.

Cell culture and cell growth assay. Human MCF-7 breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA) and maintained in MEM supplemented with penicillin, streptomycin and 10% heat-inactivated FBS in a humidified atmosphere at 37°C as previously described (15). Cells were plated at 5×10^4 cells/ml in 6-well plates under standard culture conditions. γ-Tocotrienol was dissolved in ethanol and added to the culture media to the final concentration specified in the text. The final concentration of ethanol in the culture medium during γ-tocotrienol treatment did not exceed 0.1% (v/v), and the same concentration of ethanol was added to the control cultures. After treatment, the cells were harvested, and trypan blue dye exclusion assay was used to determine the cell number using a hemocytometer.

Western blotting. For Western blotting, the MCF-7 cells were cultured as detailed above and treated with either vehicle alone or with γ-tocotrienol for 1, 2, 3 or 4 days. The cells were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail). The protein concentration of the lysates was determined by a coomassie protein assay kit (Pierce Biotechnology, Inc., IL, USA) with bovine serum albumen (BSA) as standard. Protein sample (20 μg of protein) was resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitrocellulose membrane (Whatman, Middlesex, England, UK) and then blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween 20) containing 3% nonfat dried milk overnight at 4°C. The blots were probed with specific primary antibodies as specified in the text and followed with secondary antibodies. Then, the blots were visualized by an enhanced chemiluminescent (ECL) detection system (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). In some instances the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol and 2% SDS) for 5 min at 50°C and then reprobed with other antibodies. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a ratio against the loading control.

Results

Effect of γ-tocotrienol on MCF-7 cell growth. Control of MCF-7 cell growth by γ-tocotrienol was investigated by incubating MCF-7 cells with 0, 1, 2.5, 5, 7.5 and 10 μM of γ-tocotrienol for 3 days. Cell proliferation was determined by trypan blue exclusion assay using a hemocytometer. Compared with the controls, there was a ~8%, ~23%, ~31% and 42% reduction in cell growth at 3-day exposure to 2.5, 5, 7.5 and 10 μM γ-tocotrienol, respectively (Figure 1A). Time dependent growth control by γ-tocotrienol was also studied. Cells were incubated with 10 μM γ-tocotrienol for 1, 2, 3 and 4 days. The results in Figure 1B show that while no effect on cell proliferation was evident after 1-2 days of treatment, on days 3 and 4, a ~42% and ~55% suppression of cell proliferation by 10 μM γ-tocotrienol was observed. Thus, γ-tocotrienol suppressed MCF-7 cell proliferation in a dose- and time-dependent fashion.

γ-Tocotrienol modulates specific gene expression involved in cell cycle control. Since progression through G1 checkpoint is primarily controlled by the retinoblastoma Rb/E2F protein complex, the study tested whether γ-tocotrienol affected MCF-7 cell growth by modulating the expression of Rb. Thus, levels of total Rb and Rb phosphorylated at specific amino acid residues were determined by Western blot analysis using the relevant specific Rb antibodies. Cells were treated with 10 μM of γ-tocotrienol for 1, 2, 3 and 4 days, and the changes in total and specific phosphorylated Rb expression were analyzed. Whereas no difference in total Rb was found between control and treated cells, for up to 4 days, significant changes in the phosphorylation status of Rb at specific serine and threonine residues were observed on day 1 of treatment and persisted to days 3 and 4. Specifically, immunoblot analysis showed that Rb phosphorylation at S-780 and S-807/811 was quantitatively more robust following treatment by γ-tocotrienol, compared to control cells (Figure 2A). By contrast, exposure to γ-tocotrienol reduced Rb phosphorylation T-821, particularly during days 1 and 2 of treatment (Figure 2A). Since the phosphorylation status of Rb affected the activity of transcription factor E2F, the effect of γ-tocotrienol on E2F protein expression was determined. As shown in Figure 2B, γ-tocotrienol treatment of the MCF-7 cells resulted in a modest decrease in E2F protein expression, with the most profound decrease observed after 4 days of treatment. Cyclin D1 and cdk4 are regulatory proteins known to play a pivotal role in controlling the phosphorylation status of Rb and consequently activation of transcription factor E2F for induced cell phase transition from G1 into the S-phase of the cell cycle. Therefore, the study next investigated whether exposure to γ-tocotrienol affected cyclin D1 and cdk4 levels. A marked decrease in the expression of cyclin D1 was observed in treated cells at days 1-4. Densitometric analysis showed a corresponding 50%, 84%, 92% and 94% decrease in the expression of cyclin D1 on 1-4 days of exposure to γ-tocotrienol (10 μM) (Figure 3A). The decreased cyclin D1 expression was only accompanied by a slight reduction in the expression of cdk4 (Figure 3A). To further explore whether γ-tocotrienol affected G2/M cell cycle regulatory protein expression, the changes in the expression of cyclin B1 and cdk1 were also assayed. γ-Tocotrienol caused a time-dependent decrease in the
expression of cyclin B1 and cdk1 (Figure 3B). Compared to controls, cyclin B1 showed a 60% decrease, as quantified by densitometric analysis, after 4 days of exposure to 10 μM γ-tocotrienol. In concomittance, a 42% decrease in the expression of cdk1 was found in 4 days, 10 μM γ-tocotrienol-treated cells (Figure 3B). Taken together, these results showed a loss of Rb phosphorylation at specific sites, in parallel with corresponding alterations in G1 cell cycle regulators like cyclin D1 and E2F, and further matched by a marked reduction in the expression of G2/M regulatory proteins including cyclin B1 and cdk1, as a result of exposure to γ-tocotrienol.

Copious induction of quinone reductase type 2 (NQO2) by γ-tocotrienol in MCF-7 cells. Phase II detoxification enzyme, NQO2, has been reported to catalyze the reduction of estrogen quinones and prevent the initiation of breast carcinogenesis (16). Accordingly, an assay was performed for changes in NQO2 by γ-tocotrienol in MCF-7 cells using immunoblot analysis of lysates derived from cells treated with 10 μM of γ-tocotrienol for 1, 2, 3 and 4 days. A copious induction in expression of NQO2 by γ-tocotrienol was observed on days 1-3 of treatment, corresponding to an approximately 9-, 10- and 11-fold increase, which dropped to a 6.5-fold up-regulation on day 4, when compared to the level found in day 1 of the control sample (Figure 4A). Doses dependent induction of NQO2 by γ-tocotrienol was next investigated. Cells were incubated for 3 days with 0, 1, 2.5, 5, 7.5 and 10 μM of γ-tocotrienol and the expression of NQO2 was determined. Induction of NQO2 by γ-tocotrienol was clearly evident even at 2.5 μM (Figure 4B). These findings definitively showed that γ-tocotrienol treatment results in a dose- and time-dependent increase in NQO2 expression.

Discussion

Tocotrienols have potent anticancer activities, whereas the mechanisms by which specific types of tocotrienols elicit their chemopreventive properties remain largely unknown. Using MCF-7 cells, this study provided evidence for γ-tocotrienol-elicited changes that are mechanistically tied to its anti-BCa activities: (i) γ-tocotrienol treatment reduced total Rb and its phosphorylation at the T-821 position, whereas the phosphorylation on site-specific serine residues tended to increase; (ii) γ-tocotrienol resulted in a substantial decrease in cyclin D1; (iii) γ-tocotrienol also caused a marked reduction in the expression of cdk1 involved in the G2/M checkpoint; and (iv) γ-tocotrienol induced the expression of NQO2. These results as a whole lend further support to the notion that γ-tocotrienol has a potent anti-BCa activity on MCF-7 cells, mechanistically exerting control on the expression of specific cell cycle regulatory proteins in concomittance with the suppression of cell proliferation, as well as the induction of NQO2 and thus befitting the rubric of efficacy as a chemopreventive agent.

Previously, it was shown that δ-tocotrienol inhibits estrogen receptor-negative human BCa MDA-MB-231 cell proliferation by eliciting a loss of cyclin D1 expression and associated suppression of site-specific Rb phosphorylation (17). The results of the present study showed that γ-tocotrienol suppresses estrogen receptor-positive human BCa MCF-7 cell proliferation via a similar mechanism but with slightly different mechanistic details as different site-specific Rb phosphorylation appears to be involved. Overall, however, the results of the previous and this study suggest that γ- and δ-tocotrienols both control BCa cell proliferation irrespective of the estrogen receptor status (10); however, conceivably, specific forms of tocotrienol may exert distinct preferences for different molecular targets.
Figure 2. Effect of γ-tocotrienol on Rb phosphorylation and E2F expression in MCF-7 cells. Cells were exposed to 10 μM concentration of γ-tocotrienol for 1, 2, 3 or 4 days. Cell lysates were prepared, fractionated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. A: Western blot analysis was performed for total Rb and phosphorylated Rb at S780, S807/811 and T821 positions. The blots were stripped and reprobed with actin which served as loading control. B: Western blot analysis was performed for E2F expression. The blots were stripped and reprobed with actin which served as loading control. Densitometric data of the immunoblots were normalized to a level of 1.0 based on value in control cells, adjusted using the expression of actin for each sample.

Figure 3. Effect of γ-tocotrienol on the expression of cyclin D1, cdk4, cyclin B1 and cdk1 in MCF-7 cells. Cells were exposed to 10 μM concentration of γ-tocotrienol for 1, 2, 3 or 4 days. Cell lysates were prepared, fractionated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed for cyclin D1 and cdk4 (A), and cyclin B1 and cdk1 (B) expression. The blots were stripped and reprobed with actin which served as loading control. Densitometric data of the immunoblots were normalized to a level of 1.0 present in control cells in relation to the expression of actin in each sample.
Another significant finding of the present study was evident in the robust time- and dose-dependent induction of NQO2 by γ-tocotrienol in MCF-7 cells. A recent report demonstrated that NQO2 act as a detoxification enzyme by catalyzing the reduction of estrogen quinones which could be responsible for estrogen-initiated BCa carcinogenesis (14 (16)). Estrogen quinones have been found to be substrates for NQO2 and the deactivation of carcinogenic estrogen quinones by NQO2 is faster than that of its homologue NQO1 (14). A possible implication of the observed induction of NQO2 by γ-tocotrienol may lie in its recently reported association as a susceptibility gene for BCa; a significant link has been found between the incidences of BCa and functional polymorphisms occurring within the NQO2 promoter region with an accompanying reduction in NQO2 gene expression (16). Therefore, one may surmise that NQO2 could serve both as a biomarker, as well as a novel molecular target for the chemoprevention of BCa. In this context, the observed induction of NQO2 expression in MCF-7 cells by γ-tocotrienol lends support for the chemopreventive potential of this form of vitamin E and the premise that γ-tocotrienol offers promise as a reference chemical for the development of a new family of chemopreventive agents with activity against estrogen-initiated BCa.

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**References**


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