Anti-proliferative effects of γ -tocotrienol on mammary tumour cells are associated with suppression of cell cycle progression

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Abstract

Objectives: Previous studies have shown that γ -tocotrienol induces potent anti-proliferative effects on +SA mammary tumour cells in culture; here, investigations have been conducted to determine its effects on intracellular signalling proteins involved in regulating cell cycle progression.

Materials and methods: +SA cells were maintained in mitogen-free defined media containing 0 or 4 μ M γ -tocotrienol, for 48 h to synchronize cell cycle in G₀ phase, and then they were exposed to 100 ng/ml EGF to initiate cell cycle progression. Whole cell lysates were collected at various time points from each treatment group and were prepared for Western blot analysis.

Results and conclusions: Treatment with 4 μ M γ -tocotrienol significantly inhibited +SA cell proliferation over a 4-day culture period. Moreover, this treatment resulted in a relatively large reduction in cyclin D1, cyclin dependent kinase (CDK)4, CDK2 and CDK6 levels, between 4 and 24 h after EGF exposure. Tocotrienol treatment also resulted in a relatively large increase in CDK inhibitor (CKI) p27, prior to and after EGF exposure, but had little effect on levels of CKIs, p21 and p15. Tocotrienol treatment also induced a large relative reduction in retinoblastoma (Rb) protein phosphorylation at ser780 and ser807/811. These findings strongly suggest that anti-proliferative effects of γ -tocotrienol are associated with reduction in cell cycle progression from G₁ to S, as evidenced by increased p27 levels, and a corresponding decrease in cyclin D1, CDK2, CDK4, CDK6 and phosphorylated Rb levels.

Introduction

Vitamin E is a generic term used to refer to a group of eight naturally occurring compounds that can be further subdivided into two structurally related subgroups called tocopherols and tocotrienols (1–3). While both tocopherols and tocotrienols are potent antioxidants (4), only tocotrienols display potent anti-cancer cell activity at treatment doses that have little or no effect on normal cell proliferation or viability (5,6). Specifically, treatment with γ -tocotrienol inhibits epidermal growth factor (EGF)-dependent mitogenesis in mammary tumour cells, whereas α -tocopherol does not (5–7). The EGF receptor belongs to the ErbB (mouse) or HER (human) family of receptor tyrosine kinases that include four members (ErbB1–4). When activated by their appropriate ligands, ErbB receptors form homo- and heterodimer complexes that activate downstream intracellular signalling pathways (8,9). Previous studies have shown that ErbB3 heterodimers are the most potent activators of the phosphatidylinositol-3 kinase (PI3K)/Akt mitogenic signalling pathway, and γ -tocotrienol treatment significantly decreases ErbB3 receptor tyrosine phosphorylation (activation) and subsequent downstream Akt activation (8–11).

Cell proliferation involves a series of highly regulated events that lead to DNA replication and cell division. The order of events in normal cell cycle progression includes preparation for DNA replication in Gap 1 (G₁) phase, DNA synthesis in S phase (S), preparation for division in Gap 2 (G₂) phase and cell division in mitosis (M) (12,13). Passage through these phases is regulated by cyclins and cyclin-dependent kinases (CDKs) that specifically act as regulatory binding proteins (14). Cyclin D1 forms a complex with CDK4 and/or CDK6 and together they play a critical role in modulating cell cycle progression (14). These cyclin-CDK complexes also phosphorylate and inactivate retinoblastoma (Rb) protein, which in its hypophosphorylation state, restricts cell cycle progression between G₁ to S (14). In addition, activity of cyclin-CDKs is modulated by CDK inhibitors (CKIs), including p15,

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that specifically inhibit catalytic subunits of CDK4 and CDK6, and Cip/Kip CKI family members p21 and p27, that act non-specifically to inhibit all CDKs (15).

Disruption in normal cyclin D1, CDK, CKI and hypo-phosphorylated Rb expression and/or their regulation are characteristically observed in the majority of breast tumours (16). Furthermore, EGF-dependent activation of the PI3K/Akt mitogenic pathway is intimately involved in regulating cyclin D1 expression (14,17). Taken together, these findings suggest that anti-proliferative effects of γ -tocotrienol may result from suppression in EGF-dependent cell cycle progression. Thus, our studies have been conducted to characterize the effects of γ -tocotrienol on expression and activation/inactivation of cyclin D1, CDKs, CKIs and Rb in highly malignant mouse +SA mammary epithelial cells, *in vitro*.

Materials and methods

Reagents and antibodies

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Purified γ -tocotrienol (>98% purity) was generously provided by Carotech Bhd. (Ipoh, Malaysia). Antibodies to cyclin D1, CDK2, CDK4, CDK6, p27, p15, phospho-Rb (Ser 780) and phospho-Rb (Ser 807/811) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody to p21 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody to actin (Ab-1: α , β , γ) was purchased from EMD Biosciences (La Jolla, CA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA).

Cell culture

The highly malignant +SA mammary epithelial cell line was derived from an adenocarcinoma that developed spontaneously in a BALB/c female mouse (18,19). It is characterized as being highly malignant and oestrogen-independent, and displays anchorage-independent growth when cultured in soft agarose gel. When +SA cells are injected back into the mammary gland fat pad of syngeneic female mice, they form rapidly growing anaplastic adenocarcinomas that are highly invasive and metastasize to the lung. Based on previous studies that characterized the anti-proliferative effects of γ -tocotrienol on +SA cells (20), experiments were conducted to elucidate further the effects of γ -tocotrienol on cell cycle progression in this mammary tumour cell line. Cell culture conditions have been described previously (5,18,20,21). Briefly, +SA cells were maintained in serum-free defined DMEM/F12 media containing 5 mg/ml bovine serum albumin (BSA),

10 μ g/ml transferrin, 100 U/ml soya bean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μ g/ml insulin and 10 ng/ml EGF, as a mitogen (5,18,20,21). Cells were maintained at 37.0 °C in a humidified atmosphere of 95.0% air and 5.0% CO₂. Highly lipophilic γ -tocotrienol was bound to BSA as described previously, and this stock preparation was subsequently used to prepare treatment media (5,6,22).

Experimental treatments

In cell proliferation studies, +SA cells were plated at a density of 5×10^4 cells/well in 24-well culture plates and allowed to adhere to the plastic plates overnight. The following day, cells were divided into different treatment groups, and media were replaced with fresh control or treatment media, and then returned to the incubator for 4 days culture. Cells were fed fresh control or treatment media every other day. To study the effect of treatment on +SA cell cycle progression, the method of mitogen starvation was employed, that is, using mitogen-free media to synchronize all the cells in G₁ phase of cell cycle, to prevent cell cycle phase variation between different groups before mitogen (EGF) exposure. In cell cycle signalling experiments, +SA cells were initially plated at a density of 1×10^6 cells/dish in 100 mm culture plates, and were allowed to adhere to the plastic dishes overnight. The following day, cells were divided into different treatment groups, media were removed and all cells were washed with phosphate-buffered saline to remove all traces of mitogen, then were treated with mitogen-free control or 4 μ M γ -tocotrienol treatment media, and incubated for 48 h, to allow cells to become synchronized in G₀/G₁. Subsequently, media were replaced with fresh control and treatment media containing EGF, to initiate simultaneous cell cycle progression, in all groups.

Measurement of viable cell number

The +SA viable cell number was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described previously (5,21–23). After the 4-day cell proliferation study experiments, media in all groups were replaced with fresh control medium containing 0.83 mg/ml MTT and then were incubated for 4 h. Then medium was removed; MTT crystals were dissolved in 1 ml of isopropanol and optical density of each sample was read at 570 nm on a microplate reader (SpectraCount; Packard BioScience Company, Meriden, CT, USA). Number of cells per well was calculated against a standard curve obtained by plating known concentrations of cells, as determined using a haemocytometer, at the start of each experiment.

Electrophoresis and Western blot analysis

At the end of the respective treatment periods, cells from each group were isolated using trypsin and whole cell lysates were prepared for subsequent electrophoresis and Western blot analysis, as described previously in detail (5,21–23). Protein concentration in each sample was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts (25 µg total protein/lane) of each sample were subjected to electrophoresis through 7.5% or 15% SDS–polyacrylamide minigels. All minigels within a given experiment were run simultaneously using *AccuPower model 500* power supply unit (VWR, Suwanee, GA, USA). Typically, proteins from two minigels (Figs 2–4) were transblotted at 30 V for 12–16 h at 4 °C on to a single 3'' × 4'' polyvinylidene fluoride (PVDF) membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad), according to methods of Towbin (24), and these membranes were used for subsequent Western blot analysis, as described previously (21–23,25). Briefly, membranes were blocked with TBST/2% BSA were incubated with specific primary antibodies to cyclin D1, CDK2, CDK4, CDK6, p27, p21, p15, or actin, diluted 1:5000 to

Figure 1. Effects of 0–4 µM γ -tocotrienol on the highly malignant +SA mammary epithelial cell proliferation over a 96 h treatment period. Cells in all treatment groups were initially plated (day 0) at a density of 5×10^4 cells/well in 24-well culture plates. Vertical bars indicate mean viable cell number/well $\pm$ SEM for six replicates in each treatment group. * $P < 0.05$, as compared to vehicle-treated cells in the control group.

1:10 000 in TBST/2% BSA for 2 h. However, primary antibodies to phospho-Rb (ser780) and phospho-Rb

Figure 2. Effects of γ -tocotrienol on cyclin D1 and CDK levels. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes in serum-free defined control media, to allow cells to attach. The following day they were divided into control and treatment groups, media were removed, cells were washed and then fed fresh mitogen-free media containing 0 or 4 µM γ -tocotrienol (γT^3), to establish cell cycle synchronization in all groups. After 48 h incubation period, media were removed and cells were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were then collected at 0, 2, 4, 6, 12 and 24 h after mitogen stimulation and whole cell lysates were prepared for Western blot analysis of intracellular levels of cyclin D1, CDK2, CDK4, CDK6 and actin (α , β , γ) proteins. For each experiment, samples were electrophoresed through SDS–polyacrylamide minigels run simultaneously and proteins from two minigels were transblotted on to a single PVDF membrane, followed by Western blot analysis. Each Western blot image is a representative example of data obtained from experiments that were repeated at least three times. Scanning densitometric analysis was performed for each blot and net intensity of each band was normalized with corresponding actin values, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate relative protein expression in control and treatment groups over 24 h after mitogen (EGF) stimulation.

(ser807/811) were incubated with transblotted membranes for a longer period (12 h) to optimize their binding to the corresponding protein. Membranes were washed five times with TBST and then were incubated with respective horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in TBST/2% BSA for 1 h followed by rinsing in TBST. Chemiluminescence (Pierce, Rockford, IL, USA) was used to visualize antibody-bound proteins. Images of protein bands from all treatment groups within an experiment were acquired on a single 5" × 7" film. Visualization of actin (α , β , γ) was used to ensure equal sample loading in each lane. Densitometric analysis of Western blot images was performed using Kodak Molecular Imaging Software 4.5.0 (Carestream Health Inc., New Haven, CT, USA). All experiments were repeated at least three times and a representative Western blot image from each experiment is shown in Figs 2–4. For quantification, values obtained from densitometry of Western blot images, for control and treatment groups, were normalized to their respective actin densitometric values to visualize clearly differences between treatment groups. Those values were then used to create vertical bar graphs as shown in Figs 2–4, adjacent to the corresponding Western blot image.

Statistical analysis

Differences among the various treatment groups in cell proliferation studies were determined using analysis of variance (ANOVA) followed by Dunnett's *t*-test. Difference of $P < 0.05$ was considered statistically significant, compared to vehicle-treated controls.

Results

Effects of various doses of γ -tocotrienol on proliferation of highly malignant +SA mammary epithelial cells after 4 days culture are shown in Fig. 1. Treatment with 0–4 μM γ -tocotrienol significantly inhibited cell population expansion in a dose-responsive manner. Based on these data, treatment dose of 4 μM γ -tocotrienol was selected for use in subsequent studies evaluating treatment effects on expression and activation/inactivation of intracellular proteins involved in regulating cell cycle progression.

Relative intracellular levels of cyclin D1 and CDKs in control and tocotrienol treatment groups prior to and after mitogen (100 ng/ml EGF) exposure, as determined using Western blot analysis, are shown in Fig. 2. Treatment with

Figure 3. Effects of γ -tocotrienol on CKI levels. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed serum-free defined control media to allow cells to attach. The following day, cells were divided into control and treatment groups, media were removed, cells were washed and then fed fresh mitogen-free media containing 0 or 4 μM γ -tocotrienol (γT^3), to establish cell cycle synchronization between all groups. After 48 h incubation, media were removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various time points over the next 24 h period and whole cell lysates were prepared for Western blot analysis of p21, p27, p15 and actin (α , β , γ) proteins. For each experiment, samples were electrophoresed through SDS–polyacrylamide minigels run simultaneously and proteins from two minigels were transblotted on to a single PVDF membrane, followed by Western blot analysis. Each Western blot image is a representative example of data obtained from experiments that were repeated at least three times. Scanning densitometric analysis was performed for each blot and net intensity of each band was normalized to corresponding actin values, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate relative protein expression in control and treatment groups over 24 h after mitogen (EGF) stimulation.

4 μM γ -tocotrienol resulted in significant relative reduction in cyclin D1 levels between 4 and 12 h after EGF exposure, as compared to cells in the untreated control group (Fig. 2). Tocotrienol treatment also resulted in relatively large reduction in CDK4 and CDK2 levels 4–24 h and relatively large reduction in CDK6 levels 6–24 h after EGF exposure, as compared to their respective untreated control groups (Fig. 2). Figure 3 shows Western blot analysis of treatment effects on intracellular levels of endogenous CDK inhibitors, p21, p27 and p15, prior to and after mitogen (100 ng/ml EGF) exposure. Results show that treatment with 4 μM γ -tocotrienol had little or no effect on p21 or p15 levels prior to and 24 h after EGF exposure as compared to their respective controls (Fig. 3) however, γ -tocotrienol treatment was found to cause marked increase in p27 levels prior to and up to 24 h after EGF exposure as compared to vehicle-treated controls (Fig. 3). Figure 4 shows Western blot analysis of treatment effects on intracellular levels of phosphorylated Rb protein in the cells prior to and 24 h after mitogen (100 ng/ml EGF) exposure. Treatment with 4 μM γ -tocotrienol resulted in relatively large reduction in Rb protein phosphorylation at ser780 and ser807/811 between 4 and 12 h after EGF exposure as compared to cells in the vehicle-treated control group (Fig. 4).

Discussion

Results from the present study demonstrate that anti-proliferative effects of γ -tocotrienol on these neoplastic mammary epithelial cells are associated with suppression in intracellular proteins responsible for regulating cell cycle progression. Treatment with 4 μM γ -tocotrienol resulted in relatively large decrease in EGF-dependent expression of cyclin D1, CDK2, CDK4, CDK6 and phosphorylated Rb protein. Tocotrienol treatment was also found to induce a significant increase in levels of CDK inhibitor, p27, prior to and after EGF exposure. As elevations in cyclin D1/CDK complexes, and hyperphosphorylation of Rb protein are required for transition from G₁ to S phase, these data strongly suggest that γ -tocotrienol-induced proliferation inhibition is associated with reduction in cell cycle progression from G₁ to S phase.

Cell proliferation in normal mammalian cells is a highly regulated and orderly chain of events that allows progression through phases of the cell cycle. Numerous intracellular proteins are involved in exerting positive and negative influences on this process. Cyclin expression is increased at appropriate phases in response to mitogenic signalling, and each cyclin competes with CDK inhibitors to form complexes with specific CDKs (26,27). Active

Figure 4. Effects of γ -tocotrienol on retinoblastoma (Rb) phosphorylation. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed serum-free defined control media to allow cells to attach. The following day, cells were divided into control and treatment groups, media were removed, cells washed, and then fed fresh mitogen-free media containing 0 or 4 μM γ -tocotrienol (γT^3), to establish cell cycle synchronization in all groups. After 48 h incubation, media were removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at specific times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of intracellular levels of phospho-Rb (ser780), phospho-Rb (ser807/811) and actin (α , β , γ) proteins. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and proteins from two minigels were transblotted on to a single PVDF membrane, followed by Western blot analysis. Each Western blot image is a representative example of data obtained from experiments that were repeated at least three times. Scanning densitometric analysis was performed for each blot and net intensity of each band was normalized with corresponding actin values, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate relative protein expression in control and treatment groups over 24 h after mitogen (EGF) stimulation.

cyclin-CDK complexes phosphorylate and inactivate target proteins with transcription-repressing activity, such as Rb protein. Hypophosphorylated Rb protein prevents cell cycle progression from G₁ to S phase by binding to the transcription factor, E2F, and thereby preventing gene transcription (26,27).

Defects in cell cycle restriction points that prevent progression from G₁ to S are very common in human breast cancer (28,29). Specifically, cyclin D1 characteristically displays amplification and/or overexpression in the majority of breast tumours, resulting in binding and overactivation of CDK4 and CDK6 (26,29). Amplification and/or overexpression of CDKs leads to hyperphosphorylation of Rb protein and subsequently to unregulated gene transcription and unrestricted cell cycle progression. In addition to hyperphosphorylation (inactivation) of Rb protein, many breast tumours often lack or display low expression of CDK inhibitors such as p15, p21 and p27, and these characteristics are associated with poor patient prognosis and very high patient mortality (30–32).

In the present study, mitogen-starved cells in the control group displayed rapid and sustained increase in intracellular cyclin D1 levels between 2 and 24 h after EGF exposure and this response was greatly attenuated by γ -tocotrienol treatment. In addition, γ -tocotrienol treatment resulted in sustained suppression of CDK4, CDK2 and CDK6 expression, reduction in Rb protein phosphorylation and high elevation in expression of CDK inhibitor p27, during the same period (14). As tocotrienol-induced hypophosphorylation of Rb protein would bind and prevent E2F gene transcription (30–32), these data strongly suggest that γ -tocotrienol induces cell cycle arrest between G₁ and S phase. However, additional studies are required such as flow cytometry, E2F responsive gene expression and Rb-E2F complex association to determine the specific mechanisms involved in mediating γ -tocotrienol-induced cell cycle arrest.

Oncogenic potential resulting from overexpression and/or mutation of key intracellular positive and negative regulators of cell cycle progression, is now firmly established and much effort has been taken to develop therapeutic agents that target these proteins. Results obtained in the present study strongly suggest that γ -tocotrienol might have potential to be used as a therapeutic agent, particularly since the anti-proliferative effects of this form of vitamin E are observed at doses that have little or no effect on normal mammary epithelial cell proliferation or viability (5,6). The exact molecular mechanism by which γ -tocotrienol causes down-regulation of cyclin D1 and CDKs is most likely associated with its action in suppressing EGF-dependent ErbB-receptor mitogenic signalling. Anti-proliferative doses of γ -tocotrienol have been shown

to attenuate significantly ErbB3 heterodimer activation and PI3K/Akt mitogenic signalling (20,22,25). Activated Akt phosphorylates and inactivates glycogen synthase kinase (GSK-3 β), an enzyme that negatively regulates cyclin D1 by promoting its proteosomal degradation and down-regulation (33), and previous studies have shown that treatment with 4 μ M γ -tocotrienol significantly inhibits GSK-3 β activation (20). Activation of Akt has also been shown to suppress p27 expression in cancer cells, and CDK2 activation is associated with p27 degradation (14). Therefore, tocotrienol-induced suppression of Akt and CDK2 activation would also explain increased p27 expression observed in tocotrienol-treated +SA mammary tumour cells, here.

However, the exact signalling mechanisms involved in mediating tocotrienol-induced decreases in CDK levels are not presently understood. Proliferation inhibitory doses of γ -tocotrienol may indirectly suppress transcription of CDKs or activation of certain post-transcriptional and post-translational processes that may cause decrease in CDK levels in +SA neoplastic mammary epithelial cells.

In conclusion, the present study adds significantly to existing knowledge of molecular mechanisms involved in mediating anti-proliferative effects of γ -tocotrienol in highly malignant mammary epithelial cells, *in vitro*. As γ -tocotrienol acts as a negative modulator of cell cycle progression, these findings suggest that it may have potential therapeutic value as an inducer of cell cycle arrest in treatment of breast cancer.

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