γ -Tocotrienol inhibits HGF-dependent mitogenesis and Met activation in highly malignant mammary tumour cells

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Abstract

Objectives: Aberrant Met signalling is associated with aggressive cancer cell phenotypes. γ -tocotrienol displays potent anti-cancer activity that is associated with suppression of HER/ErbB receptor signalling. Experiments were conducted to investigate the effects of γ -tocotrienol treatment on HGF-dependent +SA mammary tumour cell proliferation, upon Met activation.

Materials and methods: The +SA cells were maintained in serum-free defined media containing 10 ng/ml HGF as the mitogen. Cell viability was determined using the MTT assay, western blot analysis was used to measure protein expression, and Met expression and activation were determined using immunofluorescent staining.

Results and conclusions: Treatment with γ -tocotrienol or Met inhibitor, SU11274, significantly inhibited HGF-dependent +SA cell replication in a dose-responsive manner. Treatment with 4 μ M γ -tocotrienol reduced both total Met levels and HGF-induced Met autophosphorylation. In contrast, similar treatment with 5.5 μ M SU11274 inhibited HGF-induced Met autophosphorylation, but had no effect on total Met levels. Combined treatment with subeffective doses of γ -tocotrienol (2 μ M) and SU11274 (3 μ M) resulted in significant inhibition of +SA cell expansion compared to treatment with individual agents alone. These findings show, for the first time, the inhibitory effects of γ -tocotrienol on Met expression and activation, and strongly suggest that γ -tocotrienol treatment may provide significant health benefits in

prevention and/or treatment of breast cancer, in women with deregulated HGF/Met signalling.

Introduction

Receptor tyrosine kinases play an important role in integration and interpretation of diverse extracellular stimuli and function, to regulate various cellular processes (1,2). Met is a versatile and unique receptor tyrosine kinase that plays a role in regulating numerous biological functions including mitogenesis, motogenesis, migration, invasion and eventual metastasis (3). Met is activated by its natural ligand hepatocyte growth factor (HGF), which is also referred to as scatter factor (4). It is now well established that aberrant HGF/Met signalling plays a significant role in pathogenesis of many types of solid and haematological tumours. Binding of HGF to Met leads to receptor dimerization and tyrosine autophosphorylation of the intracellular region (5). Tyrosine phosphorylation of Met regulates internalization, catalytic activity and docking of various intracellular signalling molecules (6–8). Recent studies have shown that adaptor proteins such as Grb2, Shc, Src and the regulatory subunit of PI3K can interact directly with the activated Met receptor or indirectly through the scaffolding protein, Gab1, to stimulate downstream signalling of such pathways as PI3K, STATs and MAPK cascades (8).

In normal cells, overstimulation of Met by HGF can induce downregulation of Met as the result of receptor internalization and degradation (9). However in cancer cells, constitutive activation of Met, which can occur through mutation, overexpression and/or interaction with other cell surface receptors such as members within the HER/ErbB family of receptor tyrosine kinases, can result in unregulated Met activation and greatly enhance downstream signalling (8,10). Dysregulation of Met signalling can ultimately lead to enhanced tumour cell proliferation, resistance to apoptosis, angiogenesis, invasion and metastasis (11). Since aberrant Met signalling plays an important role in neoplastic transformation and progression, a

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great deal of interest has recently been given in targeting this receptor tyrosine kinase for treatment of metastatic cancer (12,13). Specific Met tyrosine kinase inhibitors, such as SU11274, or monoclonal antibodies directed against Met, have been shown to be effective in inhibiting Met activation, downstream signalling, and tumour cell proliferation, in various types of cancer (13).

The term 'vitamin E' represents a family of eight compounds that is divided into two subgroups called tocopherols and tocotrienols (14). Although all forms of vitamin E share the same basic chemical structure, characterized by a long phytyl chain attached to a chromane ring, tocopherols have a saturated, whereas tocotrienols have an unsaturated, phytyl chain (14). Furthermore, only tocotrienols show potent anti-cancer activity, and these effects are observed using treatment doses that have little or no effect on normal cell function or viability (15,16). It is now clearly established that γ -tocotrienol displays potent anti-proliferative and apoptotic activity in a wide variety of different cancer cell types (17–19). Recent studies have shown that the anti-proliferative effects of γ -tocotrienol are associated with suppression of EGF-dependent +SA mammary tumour cell proliferation and this effect has been found to be mediated through suppression of receptor tyrosine kinases ErbB3, ErbB4, and to a lesser extent, ErbB2 activation, and subsequent reductions in downstream PI3K/Akt and NF κ B signalling (17–22). However, effects of tocotrienols on Met receptor tyrosine kinase activation and signalling have not previously been investigated. Thus here, studies were conducted to determine whether anti-proliferative effects of γ -tocotrienol on +SA mammary tumour cells are mediated through suppression of HGF-dependent mitogenesis and Met activation.

Materials and methods

Reagents and antibodies

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Purified γ -tocotrienol was generously provided by First Tech International Ltd., (Hong Kong). Antibodies for Met (Cat #3127) and phospho-Met (Cat #3077) were purchased from Cell Signaling Technology (Beverly, MA, USA) and these antibodies had been validated by their manufacturers not to cross react with EGF receptors. Antibody to α -tubulin was purchased from EMD Biosciences (LA Jolla, CA, USA). Antibody to phospho-Met and the respective blocking peptide used for immunofluorescence studies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to EGF receptor, phospho-EGF receptor, cleaved caspase-3 and cleaved PARP were

purchased from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA). Alexa Fluor 594-conjugated anti-goat antibody, EGF, and Lipofectamine 2000 transfection reagent were purchased from Invitrogen Corporation (Carlsbad, CA, USA). HGF was purchased from Pepro-Tech Inc., (Rocky Hill, NJ, USA). Negative control scrambled siRNA and siRNA against Met were purchased from Applied Biosystems (Austin, TX, USA).

Cell line and culture conditions

Experiments conducted in the present study represent a logical extension of previous studies that have extensively characterized the anti-proliferative effects of γ -tocotrienol in neoplastic +SA mammary epithelial cells (15,23,24). The highly malignant +SA cell line itself was derived from adenocarcinoma that developed spontaneously in BALB/c female mouse (25,26). The +SA mammary tumour cell line is characterized as being highly malignant, oestrogen-independent, and displays anchorage-independent cell population growth when cultured in soft agarose gels (25). In addition, +SA cells can form adenocarcinomas that metastasize to the lungs when injected into mammary gland fat pad of syngeneic female mice (25). Cell culture conditions have been previously described in detail (15,23,24). Briefly, +SA cells were maintained in serum-free defined media containing 10 ng/ml HGF as the mitogen, Dulbecco's modified Eagle's medium (DMEM)/F12, supplemented with 5 mg/ml bovine serum albumin (BSA), 10 μ g/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μ g/ml insulin at 37 °C, in an environment of 95% air and 5% CO₂ in humidified incubator. For subculturing, cells were rinsed with sterile Ca²⁺ and Mg²⁺-free phosphate buffered saline (PBS) and incubated with 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37 °C. Released cells were centrifuged, resuspended in serum-free media and counted using haemocytometer.

Experimental treatments

To dissolve the highly lipophilic γ -tocotrienol in aqueous culture media, a stock solution of γ -tocotrienol was prepared by suspending it in a solution of sterile 10% BSA as described previously (15,23,24). Briefly, an appropriate amount of γ -tocotrienol was dissolved in 100 μ l of absolute ethyl alcohol and then added to a small volume of sterile 10% BSA in water and incubated overnight at 37 °C. This stock solution was used to prepare various concentrations of the treatment media. Ethanol was added

to all treatment groups in a given experiment such that the final concentration of ethanol never exceeds 0.1%. Stock solution of Met inhibitor, SU11274 was prepared in DMSO and DMSO was added to all treatment media such that the final concentration was same in all treatment groups and never exceeded a concentration of 0.1%.

Measurement of viable cell number

The +SA cells were initially seeded at 5×10^4 cells/well (six replicates/group) in 24-well plates in serum-free defined medium containing 10 ng/ml HGF as mitogen, and were allowed to attach overnight. For cell population growth studies, the cells were maintained in serum-free defined media containing designated doses of HGF, over a 3-day culture period. In anti-proliferative experiments, cells were exposed to respective experimental treatments containing pre-arranged concentrations of SU11274 or γ -tocotrienol alone, or in combination, for 3 days as indicated in the figure legends. For cytotoxicity studies, the cells were maintained in control serum-free defined medium containing 10 ng/ml HGF as mitogen, for 3 days. On the fourth day, cells were divided into different treatment groups and treated with the range of concentrations of SU11274 or γ -tocotrienol alone, or in combination, for 24 h. At the end of the treatment exposure period, viable +SA cell number was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described previously (15,23,24). Briefly, control and treatment media in the treatment groups in 24-well plates were replaced with fresh media containing 0.41 mg/ml MTT. After 4-h incubation period, medium was removed, MTT crystals were dissolved in isopropanol (1 ml/well), and optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company, Meriden, CT, USA). Numbers of cells/well were calculated against a standard curve prepared by plating various concentrations of cells, as determined by haemocytometer, at the beginning of each experiment.

Western blot analysis

For Western blot analysis, +SA cells were initially plated at a density of 1×10^6 cells/100-mm culture dish, allowed to attach overnight, then incubated in the respective control or treatment media. For siRNA transfection studies, 1.5×10^5 cells/well were plated in 6-well culture plates and then transfected with scrambled siRNA or siRNA against Met for 6 h, followed by incubation with media containing 10 ng/ml HGF. At the end of treatment periods, cells were isolated with trypsin, washed in PBS,

then whole cell lysates were prepared as described previously, in Laemmle buffer (27–29). Protein concentration in each sample was determined using Bio-Rad protein assay kit (Bio Rad, Hercules, CA, USA). Equal amounts of protein (30–50 μ g/lane) of each sample were subjected to electrophoresis through 7.5–15% SDS-polyacrylamide minigels. Minigels within a given treatment were run simultaneously using AccuPower model 500 power supply unit (VWR, Suwanee, GA, USA). Each gel was then equilibrated in transfer buffer and transblotted at 30 V for 12–16-h at 4 °C on to a polyvinylidene fluoride (PVDF) membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Towbin (30). These PVDF membranes were then blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) then incubated with specific primary antibodies against Met, phospho-Met, cleaved caspase-3, cleaved PARP and α -tubulin diluted 1:1000 to 1:5000 in 2% BSA in TBST for 2-h at room temperature or overnight at 4 °C. Membranes were then washed five times in TBST and incubated with respective horseradish peroxidase-conjugated secondary antibody diluted 1:3000 to 1:5000 in 2% BSA in TBST for 1-h at room temperature followed by rinsing in TBST five times. Blots were then visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Images of protein bands from all treatment groups within a given experiment were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc., New Haven, CT, USA). Visualization of α -tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least three times and a representative western blot image from each experiment is shown in figures below.

Transfection studies

Lipofectamine 2000 was used for transfection of siRNA into cells as described previously (31). Briefly, for population growth studies +SA cells were plated at concentration of 5×10^4 cells/well in 24-well plates (six replicates/group) and allowed to adhere; then cells were exposed to 240 pmol/well of scrambled or Met specific siRNA, for 6-h. After transfection, siRNA containing medium was replaced with serum-free medium containing HGF, and incubated for 3 days. Viable cells were discerned by MTT assay. For western blot analysis, cells were plated at concentration of 1.5×10^5 cells/well in 6-well plates, then exposed to 720 pmol/well scrambled or Met specific siRNA for 6-h medium was then replaced with serum-free medium containing HGF, and incubated

for 3 days. Afterwards, whole cell lysates were prepared for western blot analysis. Sense and anti-sense Met siRNA sequences (5' → 3') were CAUUUGAUUAUCACU-AUCUAtt and UAGAUAGUGAUUAUCAAUGtg, respectively.

Immunocytochemical fluorescence staining

For immunocytochemical fluorescence studies, +SA cells were plated at concentration of 1.5×10^5 cells/well (three replicates/group) in 6-well plates. After incubation in treatment media, cells in the treatment groups were rinsed in 0.05 M Tris buffered saline (TBS) pH 7.6, fixed in methanol previously cooled to -20°C and blocked with 2% donkey serum in TBS for 30 min. Cells were then incubated in phospho-Met specific primary antibody diluted 1:500, overnight at 4°C in 2% donkey serum in TBS. After washing five times with TBS, cells were incubated in the dark in Alexa Fluor 594-conjugated donkey anti-goat secondary antibody (2 $\mu\text{g}/\text{ml}$) in 2% donkey serum in TBS, for 1-h. After final five times washing, cells were embedded in Vectashield mounting medium with DAPI staining (Vector Laboratories IN., Burlingame, CA, USA). Fluorescence staining was examined using a Nikon ECLIPSE TE200-U microscope (Nikon Instruments Inc., Melville, NY, USA) coupled to a CoolSNAP *cf* CCD camera (Roper Scientific Inc., Photometrics, Tucson, AZ, USA) and digital images were captured using Nikon NIS Elements software (Nikon Instruments Inc., Melville, NY, USA). Specificity of phospho-Met antibody used in the immunocytochemical fluorescence studies was verified using blocking peptide corresponding to the epitope recognized by the primary antibody.

Statistical analysis

Differences among the treatment groups in +SA cell population growth studies were determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test. Difference of $P < 0.05$ was considered statistically significant, compared to vehicle-treated control groups or as defined in the figure legends.

Results

Effects of HGF on +SA mammary tumour cell population growth and Met receptors

Figure 1 shows +SA mammary tumour cell population growth in response to the range of doses of HGF, over a 3-day culture period. Stimulation with 1–10 ng/ml HGF significantly increased proliferation of these neoplastic

Figure 1. (a) Effects of different doses of HGF on neoplastic +SA mammary epithelial cell population growth in serum-free defined media. Cells were initially plated at a density of 5×10^4 cells/well in 24-well culture plates (six replicates/group) and treated with doses of HGF over a 3-day culture period. Viable cell number was determined by MTT colorimetric assay. Vertical bars indicate mean viable cell number $\pm$ SEM, for six replicates in each treatment group. * $P < 0.05$ compared to their respective mitogen-free control group (0 ng/ml HGF). (b) Western blot analysis of HGF-induced tyrosine phosphorylation of Met in +SA mammary tumour cells. Cells were initially plated at density of 1×10^6 cells/100 mm culture dish. Afterwards, cells were incubated overnight in mitogen-free media. Cells in all treatment groups were then stimulated with 100 ng/ml HGF for 0–60 min. Cells incubated in mitogen-free media were used as their respective controls. Following treatment exposure, whole cell lysates were prepared then subjected to polyacrylamide gel electrophoresis and western blot analysis for detection of total and phosphorylated (active) Met receptor levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times. (c) Visualization of HGF-induced activation of Met receptors using immunocytochemical fluorescence staining. Cells were initially plated at a density of 1.5×10^5 cells/well in 6-well plates (three replicates/group) then incubated in mitogen-free media overnight. Afterwards, cells were treated with 0 (mitogen-free) or 10 ng/ml HGF for 5 or 30 min. Cells were then fixed in methanol and incubated in phospho-Met specific primary antibody followed by incubation in Alexa Fluor-594 conjugated secondary antibody. Red stain indicates positive fluorescent staining for phospho-Met receptors and blue represents cell nuclei counterstained with DAPI. Magnification of each photomicrograph is 200 $\times$.

cells in a dose-responsive manner, compared to cells maintained in mitogen-free control media (Fig. 1a). HGF-induced Met receptor phosphorylation (activation) is

shown in Fig. 1b. Western blot analysis indicated that gradual decrease in total Met receptor levels occurred over a 60 min period following exposure to 100 ng/ml HGF (Fig. 1b). At the same time, HGF exposure resulted in increase in tyrosine phosphorylation (activation) of Met receptors, with maximal phosphorylation observed 30 min after HGF exposure, followed by subsequent decrease in Met receptor tyrosine phosphorylation, reflecting downregulation of Met receptors following stimulation with HGF, Met receptor's natural ligand (Fig. 1b). Immunocytochemical fluorescent staining of phosphorylated Met receptors in the cells after exposure to HGF is shown in Fig. 1c. Phosphorylated Met receptors (red) are found located both on cell membranes and in cytoplasm of the cells. Treatment with 10 ng/ml HGF for 5 or 30 min period increased fluorescence intensity of phosphorylated Met receptors, compared to cells in the mitogen-free control group (Fig. 1c). In the fluorescence images, blue cell nuclei are produced by DAPI counterstaining. In other studies, pre-exposure to blocking peptide 1-h prior to primary antibody for phosphorylated Met, resulted in almost complete absence of fluorescent staining, demonstrating antibody specificity (data not shown).

Effects of siRNA knockdown of Met receptors on population growth of +SA cells

Figure 2 shows effects of Met siRNA transfection in 10 ng/ml HGF-dependent growth of +SA mammary tumour cells after a 3-day culture period. Transfection of cells with Met-specific siRNA significantly inhibited HGF-dependent proliferation compared to cells transfected with scrambled siRNA (Fig. 2). Western blot analysis showed that cells transfected with Met-specific siRNA had high relative reduction in intracellular levels of Met compared to cells transfected with scrambled siRNA, in the presence of 10 ng/ml HGF (Fig. 2).

Effects of HGF and EGF on Met and EGF receptor activation in +SA cells

Western blot analysis shows that Met and EGF receptors in +SA mammary tumour cells did not display constitutive phosphorylation (activation) in the absence of their respective natural ligands HGF and EGF (Fig. 3). Exposure to 100 ng/ml HGF for 5 min resulted in increased tyrosine phosphorylation (activation) of Met receptors but not EGF receptors. Similarly, stimulation of the cells with 100 ng/ml EGF resulted in phosphorylation of EGF receptors but not Met receptors, reflecting absence of cross-activation and heterodimerization between EGF and Met receptors in +SA cells (Fig. 3).

Figure 2. (a) Effects of Met knockdown on HGF-dependent population growth of +SA cells. Cells were plated at density of 5×10^4 cells/well in 24-well plates then transfected with scrambled siRNA (Sc siRNA) or Met siRNA for 6-h. Afterwards, siRNA-containing medium was replaced with serum-free medium containing 10 ng/ml HGF as mitogen, and allowed to proliferate. After 3-days culture period, viable cell number was measured by MTT colorimetric assay. Vertical bars indicate mean viable cell number $\pm$ SEM in each treatment group. $*P < 0.05$ compared to scrambled siRNA treated control groups. (b) For western blot analysis, cells were plated at concentration of 1.5×10^5 cells/well in 6-well plates (three replicates/group) and then exposed to 720 pmol/well scrambled or Met specific siRNA for 6-h; then medium was replaced with serum-free medium containing 10 ng/ml HGF as mitogen then incubated for 3 days. Afterwards, whole cell lysates were prepared and subjected to polyacrylamide gel electrophoresis followed by western blot analysis for total Met levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times.

Figure 3. Total and phosphorylated (activated) levels of EGF and Met receptors in neoplastic +SA mammary epithelial cells prior to and after exposure to either EGF or HGF. Cells were initially plated at density of 1×10^6 cells/100 mm culture dish. Afterwards, cells were incubated in mitogen-free media overnight. Cells in all treatment groups were stimulated with 100 ng/ml EGF or 100 ng/ml HGF for 5 min. Cells maintained in mitogen-free media were used as untreated controls. Afterwards, whole cell lysates were prepared and then subjected to polyacrylamide gel electrophoresis and western blot analysis for total and phosphorylated (active) EGF and Met levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times.

Effects of SU11274 and γ -tocotrienol on HGF-dependent Met activation and +SA mammary tumour cell population growth

Effects of chosen doses of SU11274, a specific Met receptor tyrosine kinase inhibitor, or γ -tocotrienol on HGF-dependent +SA mammary tumour cell population growth after 3-day culture period are shown in Fig. 4a. Treatment with 4.5–5.5 μM SU11274 or 4 μM γ -tocotrienol significantly inhibited HGF-dependent cell population growth (Fig. 4a) compared to cells in the vehicle-treated control group. Effects of 5.5 μM SU11274 treatment or 4 μM γ -tocotrienol treatment on HGF-dependent Met receptor expression and activation is shown in Fig. 4b. Western blot analysis indicated that treatment with growth inhibitory concentration of SU11274 (5.5 μM) resulted in a large relative reduction in HGF-dependent Met tyrosine phosphorylation (activation), but had no effect on total intracellular levels of Met receptors compared to cells maintained in their respective vehicle-treated control groups (Fig. 4b). Alternatively, effective treatment of γ -tocotrienol (4 μM) caused significant reduction in level of total Met protein in cells as demonstrated by western blotting (Fig. 4b). Loss of Met expression caused by γ -tocotrienol treatment mirrors the loss of HGF-induced Met phosphorylation (Fig. 4b). Additional western blot studies showed that the inhibitory effect of γ -tocotrienol treatment on Met receptors follows a time-dependent

Figure 4. (a) Effects of designated doses of SU11274 or γ -tocotrienol on HGF-dependent growth of +SA cells. Cells were initially plated at density of 5×10^4 cells/well (six replicates/group) in 24-well plates and exposed to doses of SU11274 or γ -tocotrienol in the presence of 10 ng/mL HGF, throughout a 3-day culture period. Viable cell number was determined using the MTT colorimetric assay. Vertical bars indicate mean viable cell number $\pm$ SEM in each treatment group. $*P < 0.05$ compared to their respective vehicle-treated control group. (b) Effects of SU11274 or γ -tocotrienol treatment on HGF-dependent tyrosine phosphorylation (activation) of Met in +SA mammary tumour cells. Cells were plated at density of 1×10^6 cells/100 mm culture dish. They were then incubated with serum-free media containing vehicle (control), 5.5 μM SU11274 or 4 μM γ -tocotrienol and 10 ng/mL HGF as mitogen for a 3-day culture period. Following treatment exposure, cells were incubated in mitogen-free media overnight. Afterwards, cells were stimulated with 100 ng/mL HGF for 30 min. Cells incubated in mitogen-free media were used as untreated controls. Following mitogen exposure, whole cell lysates were prepared then subjected to polyacrylamide gel electrophoresis and western blot analysis for total and phosphorylated Met levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times. (c) Effects of γ -tocotrienol treatment on total levels of Met receptors in +SA mammary epithelial cells. Cells were plated at density of 1×10^6 cells/100 mm culture dish. They were then incubated in serum-free media containing vehicle (control) or 4 μM γ -tocotrienol, and 10 ng/mL HGF as mitogen. Cells were collected at various times over the next 72-h period. Following treatment exposure at each time point, cells were incubated in mitogen-free media overnight. Afterwards, they were stimulated with 100 ng/mL HGF for 30 min. Cells incubated with mitogen-free medium were used as untreated controls. Following mitogen exposure, whole cell lysates were prepared and then subjected to polyacrylamide gel electrophoresis and western blot analysis for total and phosphorylated (active) Met receptor levels. α -tubulin was visualized to ensure equal sample loading in each lane. Western blots are representative images of data obtained from experiments that were repeated at least three times. (d) Immunocytochemical fluorescence staining of phosphorylated Met receptors in +SA mammary tumour cells treated with 5.5 μM SU11274 or 4 μM γ -tocotrienol in media containing 10 ng/mL HGF. Cells were initially plated at density of 1.5×10^5 cells/well in 6-well plates (three replicates/group) then exposed to serum-free control, 5.5 μM SU11274 or 4 μM γ -tocotrienol treatment media containing 10 ng/mL HGF as mitogen for a 3-day culture period. Afterwards, cells were fixed in methanol and subjected to immunofluorescence analysis for detection of phospho-Met. Red staining indicates positive immunocytochemical fluorescence for phospho-Met receptors and blue indicates cell nuclei counter-stained with DAPI. Magnification of each photomicrograph is 200 $\times$.

course (Fig. 4c). Effective treatment with 4 μM γ -tocotrienol resulted in reduction in total levels of Met protein started after 24 h of treatment exposure, and was maximal after 3-days of culture exposure to treatment, compared to their respective vehicle-treated control groups (Fig. 4c). Further immunocytochemical fluorescent staining of the cells indicated that levels of phosphorylated Met receptors were significantly reduced when cells were treated with 5.5 μM SU11274 or 4 μM γ -tocotrienol in the presence of 10 ng/mL HGF (Fig. 4d). Treatment with 5.5 μM SU11274 or 4 μM γ -tocotrienol caused a relatively large

reduction in phosphorylated Met fluorescence intensity (red) compared to cells in their respective control groups. Blue in the fluorescent images represents cell nuclei counterstained with DAPI. In other studies, pre-exposure to blocking peptide 1-h prior to primary antibody for phosphorylated Met receptors resulted in nearly a complete absence of fluorescent staining, demonstrating antibody specificity (data not shown).

Effects of combined treatment with subeffective doses of γ -tocotrienol and SU11274 on HGF-dependent +SA mammary tumour cell population growth

Effects of combined subeffective doses γ -tocotrienol with SU11274 on HGF-dependent population growth of +SA mammary tumour cells are shown in Fig. 5a. Treatment with subeffective doses of γ -tocotrienol (2 μ M) or SU11274 (3 μ M) alone had no effect on HGF-dependent cell population growth compared to their respective vehicle-treated controls (Fig. 5a). However, combined treatment with 2 μ M γ -tocotrienol and 3 μ M SU11274 resulted in significant inhibition of cell population expansion maintained in media containing 10 ng/ml HGF, compared to control cells, and monotherapy treatment of each compound (Fig. 5a). Subsequent western blot studies show that treatment with a combination low-dose of SU11274 (3 μ M) and γ -tocotrienol (2 μ M) result in higher relative reduction in expression of Met protein associated with reduction in phosphorylated (activated) levels of Met receptors compared to cells in their respective vehicle-treated control groups, and to individual subeffective treatments of SU11274 and γ -tocotrienol (Fig. 5b).

Effects of γ -tocotrienol and SU11274 on +SA mammary tumour cell viability

Cytotoxic effects of the range of individual treatments of γ -tocotrienol (2 and 4 μ M), SU11274 (3 and 5.5 μ M) or combination treatment of both agents (2 μ M γ -tocotrienol and 3 μ M SU11274) in presence of 10 ng/ml HGF as mitogen on cell population growth are shown in Fig. 6a. Data from cell culturing showed that individual subeffective and effective treatments of γ -tocotrienol or SU11274 and combination of both agents was not associated with cytotoxic effects after 24 h of treatment exposure, compared to positive control treatment of 20 μ M γ -tocotrienol (Fig. 6a). In the cytotoxicity studies, 20 μ M γ -tocotrienol treatment is a known cytotoxic concentration in +SA tumour cells after acute exposure for 24 h in cell culture (31), and this has been used as positive control. Additional western blot studies were considered for apoptosis markers cleaved-caspase-3 and cleaved-PARP in our cells (Fig. 6b). Treatment with 20 μ M γ -tocotrienol resulted in

Figure 5. (a) Effects of combined treatment of subeffective doses of γ -tocotrienol with SU11274 on HGF-dependent +SA mammary tumour cell growth. Cells were initially plated at density of 5×10^4 cells/well (six replicates/group) in 24-well plates, and treated with 2 μ M γ -tocotrienol or 3 μ M SU11274 alone or in combination in the presence of 10 ng/ml of HGF as mitogen, for a 3-day culture period. Viable cell number was determined by the MTT colorimetric assay. Vertical bars indicate mean viable cell number $\pm$ SEM in each treatment group. * $P < 0.05$ compared to vehicle treated control groups. (b) Effects of SU11274 or γ -tocotrienol treatment on HGF-dependent expression and tyrosine phosphorylation (activation) of Met in +SA mammary tumour cells. Cells were plated at density of 1×10^6 cells/100 mm culture dish. They were then incubated in serum-free media containing vehicle (control), 3 μ M SU11274, 2 μ M γ -tocotrienol or the combination of 3 μ M SU11274 and 2 μ M γ -tocotrienol, and 10 ng/ml HGF as a mitogen for a 3-day culture period. Following treatment exposure, cells were incubated in mitogen-free media overnight and afterwards, they were stimulated with 100 ng/ml HGF for 30 min. Cells incubated with mitogen-free media were used as vehicle-treated controls. Following treatment exposure, whole cell lysates were prepared and then subjected to polyacrylamide gel electrophoresis and western blot analysis for visualization of total and phosphorylated (active) Met receptor levels. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times.

relatively high increase of cleaved caspase-3 and PARP, and served as positive control for induction of apoptosis in these cells. In contrast, treatment with subeffective doses of SU11274 and γ -tocotrienol, either alone or together had no effect on levels of cleaved caspase-3 or PARP in these cells (Fig. 6b).

Discussion

Results of this study demonstrate that HGF is a potent mitogen that stimulates proliferation of +SA mammary tumour cells in a dose-dependent manner in serum-free culture media, and this positive proliferative effect was

Figure 6. (a) Effects of γ -tocotrienol (γ -T³) and SU11274 on neoplastic +SA mammary epithelial cell viability. Cells were initially plated at density of 5×10^4 cells/well (six replicates/group) in 24-well plates and treated with serum-free defined media containing 10 ng/ml HGF as mitogen and allowed to proliferate in culture for 3 days. On the fourth day, they were divided into different groups and treated with vehicle (controls), 2 μ M γ -T³ (subeffective dose), 4 μ M γ -T³, 3 μ M SU11274 (subeffective dose), 5.5 μ M SU11274, the combination of 2 μ M γ -T³ and 3 μ M SU11274, or 20 μ M γ -T³ (cytotoxic dose) for 24 h. Treatment doses were determined based on previous findings in dose-response studies. Viable cell number was determined by MTT colorimetric assay. Vertical bars indicate mean viable cell number $\pm$ SEM in each treatment group. * $P < 0.05$ compared to vehicle treated control group. (b) For western blot analysis, cells were initially plated at a density of 1×10^6 cells/well and were allowed to replicate for 3 days in serum-free treatment media in the presence of 10 ng/ml HGF. On the fourth day, they were treated as described above for 24 h. Afterwards, whole cell lysates were prepared and subjected to polyacrylamide gel electrophoresis followed by western blot analysis for detection of cleaved caspase-3 and cleaved PARP. α -tubulin was visualized to ensure equal sample loading in each lane. Each western blot is a representative image of data obtained for experiments that were repeated at least three times.

associated with a corresponding increase in Met receptor tyrosine kinase autophosphorylation (activation). These findings are further revealed in that knockdown of Met receptors significantly attenuated HGF-dependent population growth of these cells. Furthermore, HGF treatment alone had no effect on EGF receptor phosphorylation, whereas EGF treatment alone had no effect on Met receptor activation, indicating that HGF-induced mitogenesis does not involve heterodimer cross-activation between EGF and Met receptors.

The results also indicate that anti-cancer effects of γ -tocotrienol are mediated at least in part, through suppression of Met expression and activation. Treatment with γ -tocotrienol or SU11274 (specific inhibitor of Met receptor tyrosine kinase activity), inhibited HGF-dependent Met tyrosine phosphorylation and cell population expansion in a dose-responsive manner. In addition, combined treatment with subeffective doses of γ -tocotrienol (2 μ M) and SU11274 (3 μ M) was found to significantly inhibit HGF-dependent cell population growth through a cytostatic, not cytotoxic mechanism, as indicated by the finding that combined treatment of these agents did not decrease cell viability or initiate caspase activation and apoptosis at concentrations used in these experiments. These findings show for the first time that γ -tocotrienol inhibits Met receptor expression and activation in mammary tumour cells and strongly suggest that γ -tocotrienol treatment may provide significant health benefits in prevention and/or treatment of breast cancer in women with deregulated HGF/Met signalling.

In normal mammary development, HGF is produced by mesenchymal cells and together with other growth factors acts to modulate ductal formation or tubulogenesis in a tightly controlled paracrine manner (32). However, overexpression of mutant forms of Met can result in sustained activation and neoplastic transformation and progression (32). Numerous human and murine mammary carcinoma cell lines have previously been shown to express Met and are responsive to HGF stimulation (33). Results of this study demonstrate that the highly neoplastic +SA mammary epithelial cells also express Met receptors and display HGF-dependent mitogenesis. HGF-induced Met tyrosine phosphorylation was found to reach peak levels within 30 min following mitogen exposure, whereas total Met levels displayed a gradual decrease over a 60 min HGF exposure period. HGF-induced Met receptor internalization and degradation has been shown to result from ligand-stimulated recruitment of ubiquitin ligase c-Cbl to the receptor (34). Resulting ubiquitination of Met is believed to be an essential negative regulatory mechanism that prevents excessive downstream signalling (34). Immunofluorescence studies have revealed that Met receptors in +SA mammary tumour cells are located both on the plasma membrane and in the cytoplasm. These findings confirm previous studies that described localization of Met as membranous/cytoplasmic (35).

Previous studies have shown that co-activation of Met and EGF receptors can occur in various cancer cell lines containing mutant subtypes of either receptor, that are constitutively active due to overexpression (36–38). Findings from the present study and other recent publications (20,21,24,39) provide evidence to suggest that the highly malignant +SA mammary epithelial cells express mainly

wild-type Met and EGF receptors, since neither receptor is constitutively active in the absence of their ligands, HGF and EGF, respectively. Furthermore, our results have shown that exposure to HGF alone resulted in phosphorylation and activation of Met, but not of EGF receptors, whereas exposure to EGF alone resulted in phosphorylation and activation of EGF, but not Met receptors in +SA cells. Additional studies also showed that +SA cells transfected with Met specific siRNA displayed significant suppression in HGF-dependent population growth as compared to +SA cells transfected with scrambled siRNA. These results strongly suggest an important role of both Met and EGF receptors in population growth and survival of +SA mammary tumour cells.

During the past decade, a great deal of information has been published characterizing intracellular mechanisms mediating anti-proliferative and apoptotic effects of tocotrienols in mammary tumour cells (40–43). It is now well established that treatment with relatively low doses of γ -tocotrienol significantly inhibits EGF-dependent +SA mammary tumour cell number expansion, while having no effect on cell viability (15,16). Previous studies have also shown that anti-proliferative effects of γ -tocotrienol in +SA mammary tumour cells are mediated through suppression in ErbB/HER receptor tyrosine phosphorylation and mitogenic signalling (20,21,24), strongly suggesting that γ -tocotrienol may have potential therapeutic value in treatment of breast cancers that display aberrant ErbB/HER receptor tyrosine kinase activity. Data from the present study further extend these findings and show that γ -tocotrienol is also a potent inhibitor of Met receptor tyrosine kinase activity and can effectively inhibit HGF-dependent growth of +SA cells. Furthermore, exposure to 4 μ M γ -tocotrienol causes a large reduction in total levels of Met receptors in these cells. At this time it is not known whether γ -tocotrienol-induced downregulation in Met results from decrease in gene expression and/or increase in its internalization and degradation. However, studies have shown that Met degradation is associated with increase in both proteasome and lysosomal activity, and specific inhibitors of these enzyme systems can block Met degradation (34). Additional studies are required to determine which of these mechanisms may be involved in mediating γ -tocotrienol downregulation of Met.

The tyrosine kinase inhibitor, SU11274, is 50-fold more active and selective for Met than for other tyrosine kinases (44). SU11274 acts by competing with ATP for the catalytic site on Met and downstream effects are inhibition of molecules in the PI3K pathway (44). Results in the present study show that treatment with SU11274 significantly inhibited HGF-dependent +SA cell population growth in a dose-dependent manner. In addition, SU11274 treatment inhibited HGF-dependent Met tyrosine phos-

phorylation (activation), but in contrast to γ -tocotrienol, had no effect on total Met levels. Furthermore, combined treatment with subeffective-doses of γ -tocotrienol and SU11274 significantly inhibited HGF-dependent +SA mammary tumour cell expansion and this inhibitory effect was found to be cytostatic, but not cytotoxic, since viable cell number was not reduced and the finding that PARP and caspase-3 cleavage was not observed indicates that treatments did not induce apoptosis in these cells.

At present, numerous agents have been developed as anti-cancer therapeutics that target receptor tyrosine kinases. These include monoclonal antibodies, tyrosine kinase inhibitors, immunotoxin conjugates, anti-sense oligonucleotides and specific antibodies (3,4,9,45,46). However, enthusiasm for clinical use of such agents as monotherapy has been dampened by the ability of cancer cells to escape toxicity of these drugs by forming heterodimers with other receptor tyrosine kinases. Studies have characterized cooperation between Met and HER/ErbB family of receptors in different types of cancer (47–49). Moreover, signalling networks associated with oncogenic EGF and Met receptor activation display significant overlap (47). Met amplification has been shown to promote drug resistance to gefitinib, inhibitor of the HER/ErbB receptor, by promoting heterodimer formation and mitogenic signalling with Met and/or HER3/ErbB3 heterodimers in lung cancer cells (48). Similarly, overexpression and activation of Met is associated with resistance to HER2 tyrosine kinase inhibitors in lung (49,50) and breast cancer cell lines (51,52). Recently, γ -tocotrienol treatment has been shown to potentiate anti-cancer effects of HER/ErbB tyrosine kinase inhibitors erlotinib and gefitinib in +SA mammary tumour cells (24), and these inhibitory effects were found to be associated with suppression of STAT3/5 activation and PI3K/Akt signalling (21). These findings demonstrate that combined treatment of γ -tocotrienol with other receptor tyrosine kinase inhibitors displays significantly more potent anti-cancer effectiveness than that observed with each treatment alone.

In conclusion, results in this study demonstrate that the anti-proliferative effects of γ -tocotrienol are mediated, at least in part, by suppression in Met activation. Since aberrant Met receptor activation is associated with poor prognosis in breast cancer patients, these findings strongly suggest that γ -tocotrienol may have potential therapeutic value either alone or in combination with other chemotherapeutic agents, in treatment of breast cancer in women characterized by amplified or deregulated Met mitogenic signalling.

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