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Antiproliferative effects of γ-tocotrienol are associated with lipid raft disruption in HER2-positive human breast cancer cells☆

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Abstract

A large percentage of human breast cancers are characterized by excessive or aberrant HER2 activity. Lipid rafts are specialized microdomains within the plasma membrane that are required for HER2 activation and signal transduction. Since the anticancer activity of γ-tocotrienol is associated with suppression in HER2 signaling, studies were conducted to examine the effects of γ-tocotrienol on HER2 activation within the lipid raft microdomain in HER2-positive SKBR3 and BT474 human breast cancer cells. Treatment with 0–5 μM γ-tocotrienol induced a significant dose-dependent inhibition in cancer cell growth after a 5-day culture period, and these growth inhibitory effects were associated with a reduction in HER2 dimerization and phosphorylation (activation). Phosphorylated HER2 was found to be primarily located in the lipid raft microdomain of the plasma membrane in vehicle-treated control groups, whereas γ-tocotrienol treatment significantly inhibited this effect. Assay of plasma membrane subcellular fractions showed that γ-tocotrienol also accumulates exclusively within the lipid raft microdomain. Hydroxypropyl-β-cyclodextrin (HPβCD) is an agent that disrupts lipid raft integrity. Acute exposure to 3 mM HPβCD alone had no effect, whereas an acute 24-h exposure to 20 μM γ-tocotrienol alone significantly decreased SKBR3 and BT474 cell viability. However, combined treatment with these agents greatly reduced γ-tocotrienol accumulation in the lipid raft microdomain and cytotoxicity. In summary, these findings demonstrate that the anticancer effects of γ-tocotrienol are associated with its accumulation in the lipid raft microdomain and subsequent interference with HER2 dimerization and activation in SKBR3 and BT474 human breast cancer cells.

Keywords: γ-Tocotrienol; HER2; Lipid raft; Breast cancer; Caveolin-1

1. Introduction

Breast cancer is the most prominent cancer in women and 25% of all breast cancers are characterized as having HER2 overexpression and/or excessive signaling [1]. Aberrant HER2 signaling is associated with resistance to chemotherapeutic agents and poor patient prognosis [1]. As a result, there is a great deal of interest in targeting HER2 receptors and its downstream signaling pathways in the treatment of breast cancer [2]. Although the HER2 receptor lacks a ligand binding site, HER2 activation can result by forming heterodimers with other members of the HER family of receptors [3–6]. HER2 dimerization and activation induces autophosphorylation of the intracellular tyrosine residues in the C-terminal tail, which are required for the substrate interaction, tyrosine phosphorylation and activation of multiple signaling pathways [7].

Lipid rafts play an important role in normal signal transduction in relation to the recruitment and activation of various types of receptor tyrosine kinases; such as HER family members, c-Met, VEGF and others [8,9]. However, in many types of cancer cells, lipid rafts are also involved in sequestering and concentrating oncogenic receptors to promote aberrant mitogenic signaling [8,9]. Structurally, lipid rafts represent rigid membrane microdomains that are enriched with sphingolipids and cholesterol [10,11]. In contrast to the surrounding and more fluid plasma membrane, rigid lipid rafts are enriched with specific components, such as caveolins, flotillins, nonreceptor tyrosine kinases, glycophosphatidylinositol-anchored proteins, protein kinase C and palmitoylated proteins [10,11]. Together, these components form an ordered, tightly packed rigid microdomain that is resistant to detergent solubilization and can thereby be isolated from the surrounding plasma membrane subcellular fraction [10,11].

Lipid rafts can be classified into two different subgroups that share common lipid moieties but differ in their protein composition and structural shape. The first subgroup is classified as the caveolar lipid raft and is characterized as having a flask invagination shape and is enriched with caveolins coating proteins [12]. Caveolar lipid rafts facilitate the recruitment and proximity of receptor tyrosine kinases to enhance dimerization, activation and downstream signaling in cancer cells [13]. Recent studies have shown that chronic disruption of lipid rafts integrity can lead to a reduction in cancer cell survival [14], migration and metastasis [15] and the initiation of apoptosis [16,17]. The second subgroup is classified as the noncaveolar lipid raft and is characterized as having a flat shape that lacks caveolins proteins but is

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enriched with flotillins coating proteins [12]. However, the preponderance of evidence suggests that noncaveolar lipid rafts do not appear to play a significant role in receptor tyrosine kinase recruitment, activation and signaling [12].

γ-Tocotrienol is a member of the vitamin E family of compounds that displays potent anticancer activity at treatment doses that has little or no effect on normal cell viability [18]. Previous studies have shown that the anticancer effects of γ-tocotrienol are associated with the suppression of HER2 mitogenic signaling, particularly the PI3K/Akt/mTOR [19–21], JAK/Stat [22] and MAPK pathways [23]. However, the exact cellular/molecular target involved in mediating these inhibitory effects of γ-tocotrienol has not yet been identified. Since γ-tocotrienol displays a wide range of inhibitory effects on a variety of receptor tyrosine kinases and lipid raft microdomains recruit and activate various receptor tyrosine kinases in a wide range of cancer cell types, it is possible that γ-tocotrienol might accumulate in the lipid raft microdomains of cancer cells to interfere with receptor tyrosine kinase dimerization, activation and signaling. To investigate if this hypothesis is correct, studies were conducted to determine if the inhibitory effects of γ-tocotrienol on HER2 receptor activation and signaling in HER2-positive SKBR3 and BT474 human breast cancer cells are associated with a disruption in lipid raft integrity and function.

2. Materials and methods

2.1. Reagents and antibodies

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Purified γ-tocotrienol was generously provided by First Tech International Ltd. (Hong Kong). The molecular probe DilC16 and cross-linker dithiobis succinimidyl propionate (DSP) were purchased from Life Technologies (Grand Island, NY, USA). Antibodies for cleaved caspase-3, cleaved PARP, HER2 receptor, phospho-HER2, β-actin and caveolin-1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for α-tubulin was purchased from Calbiochem (San Diego, CA, USA). Alexa Fluor 594-conjugated antigoat antibody and EGF were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

2.2. Cell culture conditions

SKBR3 and BT474 are human breast cancer cells that characteristically overexpress HER2 and were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 serum-free defined media containing 10 ng/ml EGF as a mitogen that was supplemented with 5 mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 μg/ml insulin. All cells were maintained at 37°C in an environment of 95% air and 5% CO_2 in a humidified incubator. For subculturing, cells were rinsed in a sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 4 min at 37°C. Released cells were centrifuged, resuspended in medium and counted using a hemocytometer.

2.3. Experimental treatments

γ-Tocotrienol is a highly lipophilic compound that is insoluble in water. In order to prepare a stock solution of γ-tocotrienol, a known amount of γ-tocotrienol was dissolved in 100 μl of absolute ethanol and then added to a small volume of sterile 10% BSA in water and incubated at 37°C overnight with gentle shaking, as previously described [21,24]. This stock solution was used to prepare various concentrations of the treatment media. Stock solution of 2-hydroxypropyl-β-cyclodextrin (HPβCD) was prepared in sterile distilled water.

2.4. Measurement of viable cell number

In cell growth studies, SKBR3 and BT474 cells were initially seeded at a density of 1×10^4 cells/well in 96-well culture plates (6 replicates/group) in serum-free defined control media containing 10 ng/ml EGF and allowed to attach overnight. The following day, cells were divided into different treatment groups and then given defined media that contain various concentrations of γ-tocotrienol and/or drug treatment and fed fresh treatment media every other day for 5 days. In cytotoxicity experiments, SKBR3 and BT474 cells were maintained in defined media containing 10 ng/ml EGF for 5 days (approximately 80% confluence). The next day, cells were divided into different treatment groups and treated with 0–10 mM HPβCD for 45 min on ice and then washed three times, fed fresh defined media containing 0–40 μM γ-tocotrienol and returned to the incubator for a 24-h culture period. Afterward, viable cell number was determined using the 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously [21,24,25]. Briefly, the media in various treatment groups were replaced with fresh media containing 0.5 mg/ml MTT. After a 3-h incubation, media were removed and MTT crystals were dissolved in DMSO (100 μl/well for 96-well plates). Optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount; Packard BioScience Company, Meriden, CT, USA). The number of cells per well was calculated against a standard curve prepared by plating known cell densities, as determined by hemocytometer, in triplicate at the beginning of each experiment.

2.5. Triton X-100 extraction of lipid raft

Extraction of lipid raft microdomains with triton X-100 was performed as previously described [26]. After incubation in treatment media, SKBR3 and BT474 cells in the various treatment groups were isolated by centrifugation and then resuspended in a 3-ml buffer composed of 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) and 150 mM NaCl (pH 6.5). An additional 3 ml of the same buffer plus 2% triton X-100, 2 mM Na_3VO_4 and 2 mM phenylmethylsulfonyl fluoride (PMSF) was then added to all cell suspensions and incubated on ice for 30 min. The insoluble lipid raft microdomain fractions were then pelleted by centrifugation at 14,000*g* for 20 min at 4°C. The soluble supernatant was removed and designated as the nonlipid raft soluble "NR" fraction. Afterward, the lipid raft pellets were resuspended in a different buffer composed of 1% triton X-100, 10 mM Tris (pH 7.6), 500 mM NaCl, 2 mM Na_3VO_4, 60 mM β-octylglucoside and 1 mM PMSF and incubated for 30 min on ice. The solubilized lipid raft "R" fraction was then separated as a supernatant from the cellular debris by centrifugation at 14,000*g* for 20 min at 4°C.

2.6. Cross-linking of HER2 receptor

Cross-linking of dimerized HER2 receptors was performed using a modification of the method described by Adachi *et al.*[27]. Following experimental treatment, SKBR3 and BT474 cells were washed with ice-cold PBS and then resuspended in 1.5 ml with the DSP cross-linking reagent that was composed of 3 mM DSP in 25 mM Na_2PO_4 (pH 7.4) and incubated on ice for 40 min. The reaction was quenched by the addition of 20 mM Tris (pH 7.4) in PBS. The cells were then washed twice with cold PBS and lysed in a buffer containing 50 mM/L Hepes (pH 7.5), 10% glycerol, 0.5% triton X-100, 1.5 mM/L $MgCl_2$, 1 mM/L EDTA, 1 mM/L Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 mM/L PMSF and were resuspended by aspiration through a 23-gauge needle. The lysates were then centrifuged at 14,000 rpm for 10 min at 4°C. The collected supernatant was then used for subsequent western blot analysis.

2.7. Lipid raft purification and HPLC detection of γ-tocotrienol

Lipid raft fractions were purified using sucrose gradient ultracentrifugation [28]. Following a 5-day treatment period with 0–4 μM γ-tocotrienol, cells were washed with cold PBS and then resuspended in 1 ml buffer containing 1% triton X-100, 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate and 1 mM PMSF (TNEV buffer). The suspensions were then homogenized with 10 strokes using a glass potter (Kontes, Vineland, NJ, USA) and then centrifuged at 1000 rpm for 8 min. Afterward, 1 ml of the supernatant was mixed with 1 ml of 85% sucrose in TNEV buffer at the bottom of the 10.4-ml ultracentrifuge tube, overlaid gently with 35% and 5% sucrose in TNEV buffer. Finally, all samples were centrifuged at 55,000 rpm for 18 h at 4°C. Nine 1-ml fractions were collected from the top to the bottom of the sample tube and labeled. To determine the localization of lipid rafts in these nine fractions, 50 μl from each fraction was subjected to western blot analysis for caveolin-1. The localization of γ-tocotrienol in these fractions was determined by HPLC. Briefly, 1 mg of raft and nonraft fractions was extracted using ethyl acetate and dried under nitrogen gas and then dissolved in a 1-ml HPLC-grade methanol. The resultant clear solution was then injected into Dionex HPLC system (Dionex, Sunnyvale, CA, USA) containing a 250 mm×4.6 mm Luna 5μ column (Phenomenex, Torrance, CA, USA), ASI-100 automated sampler and 3000 FLD fluorescence detector. The column was eluted with a mobile phase consisted of methanol, ethanol and acetonitrile (85:7.5:7.5 v/v/v) delivered at a flow rate of 1.0 ml/min at an injection volume of 10 μl. γ-Tocotrienol levels was detected using fluorescence detection set at 296 nm excitation and 330 nm emission with 10 min total runtime and the retention time for γ-tocotrienol was determined to be 3.8 min [29,30]. In cytotoxicity study, SKBR3 and BT474 cells were maintained in control serum-free defined media containing 10 ng/ml EGF as a mitogen for a 5-day culture period. Afterward, cells were divided into different treatment groups and treated with 0 or 3 mM HPβCD for 45 min on ice, washed three times and then fed fresh defined media containing 0 or 20 μM γ-tocotrienol for an acute 24-h treatment period. Cells were then prepared for lipid raft purification and HPLC detection of γ-tocotrienol as described above.

2.8. Western blot analysis

SKBR3 and BT474 cells were isolated with trypsin and then whole cell lysates were prepared as previously described [21]. Protein concentration in each sample was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). An equal amount of each sample was subjected to electrophoresis through SDS-polyacrylamide minigels. Proteins from gels were transblotted at 30 V for 12–16 h at 4°C onto a single