Green Tea Polyphenol Epigallocatechin-3 Gallate Inhibits Her-2/Neu Signaling, Proliferation, and Transformed Phenotype of Breast Cancer Cells

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Abstract

Overexpression of the epidermal growth factor receptor family member Her-2/neu in breast cancer is associated with poor prognosis. With evidence accumulating for a chemopreventive role of green tea polyphenols, the effects of epigallocatechin-3 gallate (EGCG) on Her-2/2-neu-overexpressing breast cancer cells were examined. EGCG inhibited mouse mammary tumor virus (MMTV)-Her-2/2-neu NF639 cell growth in culture and soft agar. EGCG reduced signaling via the phosphatidylinositol 3-kinase, Akt kinase to NF-\(\kappa\)B pathway because of inhibition of basal Her-2/2-neu receptor tyrosine phosphorylation. EGCG similarly inhibited basal receptor phosphorylation in SMF and Ba/F3 2 + 4 cells, which suggests the potential beneficial use of EGCG in adjuvant therapy of tumors with Her-2/neu overexpression.

Introduction

The Her-2/neu (or c-erbB-2) oncogene, which is the second member of the EGFR\(^1\) family (EGFR-2), encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu, which has been seen in \(\sim 30\%\) of breast cancers, is associated with poor overall survival (1). In particular, it has been found associated with increased metastatic potential and resistance to chemotherapeutic agents. Transgenic mice overexpressing Her-2/neu develop focal mammary tumors (2). Recent work has implicated the PI 3-kinase to serine/threonine kinase Akt/protein kinase B to NF-\(\kappa\)B and soft agar. EGCG reduced signaling via the phosphatidylinositol 3-kinase, Akt kinase to NF-\(\kappa\)B pathway because of inhibition of basal Her-2/2-neu receptor tyrosine phosphorylation. EGCG similarly inhibited basal receptor phosphorylation in SMF and Ba/F3 2 + 4 cells, which suggests the potential beneficial use of EGCG in adjuvant therapy of tumors with Her-2/neu overexpression.

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\(^4\) The abbreviations used are: EGFR, epidermal growth factor; EGFR, EGFR receptor; PI 3-kinase, phosphatidylinositol 3-kinase; EGCG, epigallocatechin-3 gallate; MTX, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PMSF, phenylmethylsulfonyl fluoride; LP, leupeptin; WCE, whole cell extract; EMSA, electrophoretic mobility shift assay; DMBA, 7,12-dimethylbenz(o)anthracene; NF, nuclear factor; MMTV, mouse mammary tumor virus; GSK3\(\alpha\), glycogen synthase kinase 3\(\alpha\).
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Results

EGCG Inhibits Growth of MMTV-Her-2/neu Breast Cancer Cell Lines. We first tested the ability of EGCG to inhibit growth of the MMTV-Her-2/neu mouse breast tumor derived cell line NF639. Cultures were plated, in triplicate, at a density of $3.9 \times 10^3$ cells/cm². After 24 h, EGCG was added to a final concentration of 20–160 µg/ml, or the volume of carrier DMSO equivalent to the highest dose was added. Cell growth was assessed every 24 h using a nonradioactive MTS cell proliferation assay (Fig. 1). A dose-dependent decrease in the rate of proliferation was seen with 20 and 40 µg/ml EGCG or carrier DMSO solution for 24 h. Cells were harvested and extracts assayed for luciferase activity as described previously (5). Averages values are presented ± SD. SD was obtained using the Student’s t test.

Focus Formation Assay. NF639 cells were plated, in triplicate, at 7.5 $\times 10^3$/ml in top plugs consisting of complete medium and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, ME) with the indicated concentration of EGCG. Plates were subsequently incubated for 2 weeks in humidified incubator at 37°C. Cells were stained with 2 ml of crystal violet solution and washed extensively with water, and colonies were counted using an inverted bright field microscope at a ×2 magnification. Three random fields were counted from each of the triplicate samples, and average values presented ± SD, which was determined in each set of nine values obtained using the Student’s t test.

Transfection Analysis. Twenty-four h after plating at 30% confluence in P150 dishes, NF639 cells were transfected with 5 µg of NF-kB-element-driven luciferase reporter DNA (10), kindly provided by Georges Rawadi (Hoescht-Marion-Roussel, Romainville, France) with 25 µg of pcDNA empty vector in 45 µl of FuGENE Reagent. Three h after transfection, cells were trypsinized and replated in multiple P60 dishes. After overnight incubation, cells were treated in duplicate with either 50 µg/ml EGCG or carrier DMSO solution for 24 h. Cells were harvested and extracts assayed for luciferase activity as described previously (5). Averages values are presented ± SD. SD was obtained using the Student’s t test.

EGCG inhibits growth in soft agar, an important property of the transformed phenotype. Hence, we next assessed the ability of EGCG to reduce growth of NF639 cells in soft agar. NF639 cells were plated, in triplicate, in soft agar at a density of $7.5 \times 10^3$ cells/ml in the absence or presence of the indicated concentration of EGCG. After 2 weeks, colonies were stained and three random fields counted from each of the triplicate samples. Average percentage of control colony numbers per field ± SD presented as a function of EGCG concentration. Inset, stained colonies were photographed using a Kodak digital camera.

EGCG Reduces NF-κB Activity in the NF639 Cell Line. To assess the effects of EGCG on Her-2/neu signaling, we first monitored the recently identified downstream target NF-κB (5). NF639 cells were treated with 40 µg/ml EGCG for 24 h and nuclear extracts prepared. These were used in EMSA with an oligonucleotide containing the NF-κB element upstream of the c-myc promoter. Extracts from DMSO-treated cells gave rise to two bands (Fig. 3A). These have been identified previously as containing complexes composed of p65/p50 and p50 homodimers (p50/p50). Incubation in the presence of 40 µg/ml EGCG reduced formation of both of these two complexes. This decrease was selective, because no change was seen in Sp-1 binding (Fig. 3A). We next assessed the effects of EGCG on NF-κB activity. A transfection assay was performed using an NF-κB element-driven luciferase reporter. In two experiments, treatment with 50 µg/ml EGCG for 24 h reduced NF-κB activity by 76.3% ± 5.9%. Thus, EGCG inhibits NF-κB binding and activity in NF639 cells.

EGCG Inhibits the PI 3- to Akt Kinase Signaling Pathway. Previously, we showed that induction of NF-κB in NF639 cells occurs via a PI 3-kinase-to-Akt kinase pathway (5). To test the effect of EGCG treatment on Akt phosphorylation, the cells were incubated for 24 h in the presence of either 40 µg/ml of EGCG that had been dissolved in DMSO or the equivalent amount of carrier solution. Alternatively, cells were incubated in the presence of 100 nm Wortmannin, a potent specific inhibitor of PI 3-kinase (11). WCEs were prepared and immunoprecipitated with a monoclonal anti-Akt, which preferentially recognizes phosphorylated protein. GSK3α-GST was then used as the substrate for the resulting immunoprecipitated Akt, and the phosphorylated material identified by immunoblot analysis for phosphorylated GSK3α-GST protein (Fig. 3B). The EGCG treatment
This effect was general, we analyzed two additional cell lines: (a) SMF/H9262 and (b) Ba/F3 2 + 4 cells, a pro-B cell line clone stably expressing Her-2/neu + EGFR-4 (5, 9), displaying high constitutive Her-2/neu activity (5). SMF and Ba/F3 2 + 4 cells were treated with 40 μg/ml EGCG for 24 h and analyzed by immunoblotting for phosphorylated Her-2/neu (Fig. 4B). EGCG treatment similarly reduced basal phosphorylation of Her-2/neu in both cell lines. Thus, EGCG reduces the basal phosphorylation and constitutive activation of the Her-2/neu receptor.

Discussion

Here we show that the green tea polyphenol EGCG inhibits the signaling by Her-2/neu that promotes cell proliferation, survival, and transformed phenotype. Treatment of MMTV-Her-2/neu mammary gland tumor NF639 cells with doses of EGCG up to 80 μg/ml slowed growth and dramatically reduced colony formation in soft agar with little effect on cell viability. Higher doses (160 μg/ml) reduced cell numbers and induced cell death as judged by trypan blue assays. Previously, we demonstrated that the overexpression of Her-2/neu in NF639 cells leads to the induction of NF-κB via a PI 3-kinase/Akt kinase signaling pathway (5). EGCG inhibited this Her-2/neu signaling as judged by the observed decreases in PI 3-kinase, Akt kinase, and NF-κB activities. Furthermore, EGCG inhibited constitutive Her-2/neu phosphorylation in NF639 cells, as well as in SMF cells, a second mouse MMTV-Her-2/neu tumor-derived cell line, and Ba/F3 2 + 4 cells, a pro-B cell line transfected to express Her-2/neu + EGFR-4. Overall, these studies demonstrate for the first time that EGCG can ablate the Her-2/neu signaling cascade in breast cancer cells, by reducing basal Her-2/neu receptor phosphorylation. These results suggest further study of the potential role of EGCG in adjuvant therapy treatment modalities and of green tea components in chemoprevention of breast cancer are warranted.

In animal models, green tea extracts have been shown to inhibit chemical and photo carcinogenesis. For example, green tea was found to inhibit cancers of the gastrointestinal tract, lung, and skin in mice.

resulted in a dramatic decrease in phosphorylated GSK3α-GST. A similar decrease was observed with Wortmannin treatment.

As a test for the effects of EGCG on PI 3-kinase activity, we measured the presence of phosphorylated Akt, which is one of its critical signaling substrates. Immunoblotting was performed using an antibody specific for phospho-Akt (Ser473). Treatment with EGCG caused an ~50% decrease in phosphorylated Akt protein (Fig. 3C). A similar level of decrease was observed on treatment with Wortmannin, which suggested that the remaining phosphoprotein detected is attributable to other kinase activities. Immunoblotting for β-actin confirmed equal loading. Together, these results demonstrate the ability of EGCG to reduce the activities of both PI 3-kinase and Akt.

EGCG Reduces Basal Phosphorylation of Her-2/neu. Overexpression of Her-2/neu can lead to constitutive phosphorylation and basal receptor activation and signaling. Thus, we tested whether treatment with EGCG can reduce this basal Her-2/neu phosphorylation. NF639 cells were treated with 0, 20, 40, or 80 μg/ml EGCG for 48 h. WCEs were isolated and subjected to immunoblot analysis using a tyrosine phosphospecific Her-2/neu antibody (Fig. 4A). A dose-dependent drop in phosphorylated Her-2/neu protein was noted. Densitometry indicated treatment with 20, 40, or 80 μg/ml caused a decrease of 13, 38, and 96%, respectively compared with control cells. Immunoblotting for β-actin confirmed equal loading. To verify that this effect was general, we analyzed two additional cell lines: (a) SMF cells, similarly derived from an MMTV-Her-2/neu mouse mammary tumor (8); and (b) Ba/F3 2 + 4 cells, a pro-B cell line clone stably expressing Her-2/neu + EGFR-4 (5, 9), displaying high constitutive Her-2/neu activity (5). SMF and Ba/F3 2 + 4 cells were treated with 40 μg/ml EGCG for 24 h and analyzed by immunoblotting for phosphorylated Her-2/neu (Fig. 4B). EGCG treatment similarly reduced basal phosphorylation of Her-2/neu in both cell lines. Thus, EGCG reduces the basal phosphorylation and constitutive activation of the Her-2/neu receptor.
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(reviewed in Ref. 6). It has been reported also that EGCG inhibits the growth of human breast and prostate tumors transplanted into athymic mice (12). We recently demonstrated that green tea extracts given to female Sprague Dawley rats in their drinking fluid significantly decrease DMBA-induced mammary tumor burden and invasiveness and significantly increase latency to first tumor (7). Another study using a diet containing 1% green tea catechins fed to female Sprague Dawley rats showed that tea was effective in reducing mammary gland tumorigenesis in the promotion, but not the progression, stages of carcinogenesis (13). Consistent with these findings, several studies have also found that green tea treatment of breast cancer cells in vitro reduces their rate of proliferation (7, 14, and references therein). Overall, the toxicity of green tea extracts is low, and, thus, they represent potentially useful cancer chemopreventive agents.

Here, EGCG was shown to reduce NF-κB levels and activity in NF639 cells because of its ability to inhibit the Her-2-neu signaling pathway that leads to NF-κB activation. Further insight into the regulatory mechanisms will be provided when the endogenous downstream targets of NF-κB responding to EGCG treatment are elucidated. Our findings are consistent with previous observations on the ability of EGCG to block signaling by the EGFR family (15, 16). For example, Liang et al. (15) demonstrated the ability of EGCG to block EGF signaling via the EGFR in A431 epidermoid carcinoma cells. Interestingly, we observed cooperative inhibition of NF-κB binding levels on EGCG cotreatment with an antibody against the Her-2-neu receptor.5 Yang et al. (17) observed that green tea polyphenols, including EGCG, could inhibit activated IκB kinase in the intestinal epithelial cell line IEC-6. Green tea extracts have also been found to block NF-κB activation in cancer cells on tumor necrosis factor-α, lipopolysaccharide, or UV treatment (18, 19). In contrast, EGCG failed to reduce NF-κB levels in Hs578T breast cancer cells6 that were derived from a carcinosarcoma and display constitutive IκB receptor.5 Yang levels on EGCG cotreatment with an antibody against the Her-2/neu signaling pathway display variable sensitivities to EGCG. Of note, the basal activation of Her-2/neu seems sensitive to EGCG inhibition.

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References


5 S. P., unpublished observations.

6 K. T. K, unpublished observations.
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