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/neu-overexpressing breast cancer cells were examined. EGCG inhibited mouse mammary tumor virus (MMTV)-Her-2/neu NF639 cell growth in culture and soft agar. EGCG reduced signaling via the phosphatidylinositol 3-kinase, Akt kinase to NF-kB pathway because of inhibition of basal Her-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 (c-erbB-2) oncogene, which is the second member of the EGFR family (EGFR-2), encodes a transmembrane tyrosine receptor kinase. Overexpression of Her-2/neu, which has been seen in ~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-kB signaling pathway in control of growth and transformed phenotype of Her-2/neu-overexpressing cells (2–3). Green tea is rich in polyphenols, such as EGCG, that possess antioxidant qualities, which have been shown to have anticarcinogenic activity against a variety of tumor types including breast cancer (reviewed in Ref. 6). For example, we recently showed that female rats given green tea as their drinking fluid displayed a significant decrease in carcinogen-induced mammary tumor burden and invasiveness and significantly increased latency to first tumor (7). Doses of either green tea polyphenol mix or EGCG between ~40 to 80 μg/ml slowed growth of various estrogen receptor-negative breast cancer cell lines in culture (7). Furthermore, statistics indicate that the incidence of breast cancer in regions in which green tea is consumed in large quantities, including China and Japan, is much lower than in western societies. Here we have examined the effects of EGCG on Her-2/neu-overexpressing breast cancer cells. We report that treatment of MMTV-Her-2/neu mouse mammary tumor NF639 cells with EGCG slows proliferation and reduces growth in soft agar via inhibiting the PI 3- to Akt kinase to NF-kB pathway. EGCG-reduced basal receptor tyrosine phosphorylation in NF639 and two other Her-2/neu-overexpressing lines. These findings suggest the use of EGCG may be beneficial in chemoprevention of breast cancer and represents a possible tool in adjuvant therapy modalities of patients with tumors overexpressing the Her-2/neu receptor.

Materials and Methods

Cell Growth and Treatment Conditions. The MMTV-Her-2/neu cell lines NF639 and SMF (kindly provided by P. Leder, Harvard Medical School, Boston, MA) were derived from mammary gland tumors and cultured as described previously (8). Ba/F3 pro-B cells, which are transfected to express Her-2/neu and EGFR-4 (Ba/F3–2+4), were kindly provided by David Stern (Yale University, New Haven, CN; Ref. 9). These cells, which display high constitutive Her-2/neu phosphorylation (5), were grown in RPMI supplemented with 10% fetal bovine serum, conditioned medium from WEHI 3 cells, and antibiotics, as described previously (9). EGCG, purchased from LKT Laboratories Inc. (St Paul, MN), was dissolved in sterile 50% DMSO at a concentration of 100 mg/ml, and diluted with double-distilled H2O to a 1 mg/ml working-strength solution. Nonradioactive MTS cell proliferation assays (Promega) were performed essentially as we have published previously (7).

EMSA. Nuclear extracts were prepared from breast cancer cells in extraction buffer [420 mM KCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol] plus protease inhibitors (0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml LP), as we have published previously (5). The sequence of the URE NF-kB-containing oligonucleotide from the c-myc gene is as follows: 5'-GATCCAGTGCAGGTTTTCCACCC-3', in which the underlined region indicates the core binding element. The sequence of the Sp1 oligonucleotide is 5'-ATTCGATCGGGCGGGCGCACC-3'. Oligonucleotides were end labeled with large Klenow fragment of DNA polymerase and [γ-32P]ATP. The EMSA was performed using 5 μg of nuclear extract, essentially as we have published previously (7).

Akt Kinase Assay. The Akt kinase assay, was performed following the directions of the Akt Kinase Assay kit (New England Biolabs, Beverly, MA). Briefly, samples (100-μg) of WCEs were immunoprecipitated overnight with an agarose conjugated anti-Akt antibody (New England Biolabs). The immunoprecipitate was resuspended in kinase buffer, and the assay performed at 30°C for 45 min, using 1 μg of GSK3s-GST fusion protein as substrate in the presence of 10 μM ATP. The resulting products were resolved in a 10% polyacrylamide-SDS gel and subjected to immunoblotting, as below, using phosphospecific GSK3s antibody (New England Biolabs).

Immunoblot Analysis. Cells were rinsed with cold PBS, and harvested in lysis buffer [50 mM Tris-HCl ([pH 8.0]), 5 mM EDTA ([pH 8.0]), 150 mM NaCl, 0.5 mM DTT, 2 μg/ml aprotinin, 2 μg/ml LP, 0.5 mM PMSF, 0.5% and NP40]. WCEs were obtained by sonication, followed by centrifugation at 14,000 rpm for 30 min. Samples were subjected to electrophoresis in a 10% polyacrylamide-SDS gel and immunoblotting, as previously described (5). Antibodies used were: phosphorylated Akt Ser473 (Cell Signaling) and tyrosine phospho-
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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 \times 2 magnification. Three random fields were counted from each of the triplicate samples, and average values presented \( \pm \) SD, which was determined in each set of nine values obtained using the Student\’s t test.

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 10^4 cells/cm^2. After 24 h, EGCG was added to a final concentration of 20–160 \( \mu \)g/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 \( \mu \)g/ml EGCG, whereas no cell growth was seen with 80 or 160 \( \mu \)g/ml EGCG. On treatment with 160 \( \mu \)g/ml EGCG, a drop in cell numbers was seen. This suggested that extensive death of NF639 cells was occurring only at this higher dose, which was confirmed by trypsin blue staining of cells with 0, 40, 80, or 160 \( \mu \)g/ml EGCG. EGCG treatment on Akt phosphorylation, the cells were incubated for 24 h reduced NF-\( \kappa \)-B (5). NF639 cells assessed the ability of EGCG to reduce growth of NF639 cells in soft agar, a hallmark of transformed phenotype. Hence, we next

Fig. 1. EGCG inhibits growth of NF639 cells. NF639 cells were plated, in triplicate, at a density of 3.9 \times 10^4 cells/cm^2. After overnight incubation, EGCG was added at the indicated concentration (\( \mu \)g/ml) dissolved in DMSO or with the volume of DMSO vehicle alone equivalent to the highest dose used, as control (0 \( \mu \)g/ml). Cultures were incubated for an additional 24, 48, or 72 h. Cell proliferation was quantified using the nonradioactive proliferation assay as measured by conversion of MTS dye to its formazan product read at 490 nm. The data are presented as the mean \( \pm \) SD.

EGCG Inhibits Growth of NF639 Cells in Soft Agar. Growth in soft agar is a hallmark of transformed phenotype. Hence, we next

Fig. 2. EGCG inhibits 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 \( \pm \) SD presented as a function of EGCG concentration. Inset, stained colonies were photographed using a Kodak digital camera.

EGCG reduces NF-\( \kappa \)-B 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-\( \kappa \)-B (5). NF639 cells were treated with 40 \( \mu \)g/ml EGCG for 24 h and nuclear extracts prepared. These were used in EMSA with an oligonucleotide containing the NF-\( \kappa \)-B 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, 5). Incubation in the presence of 40 \( \mu \)g/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-\( \kappa \)-B activity. A transfection assay was performed using an NF-\( \kappa \)-B element-driven luciferase reporter. In two experiments, treatment with 50 \( \mu \)g/ml EGCG for 24 h reduced NF-\( \kappa \)-B activity by 76.3% \( \pm \) 5.9%. Thus, EGCG inhibits NF-\( \kappa \)-B binding and activity in NF639 cells.

EGCG inhibits PI 3- to Akt kinase signaling pathway. Previously, we showed that induction of NF-\( \kappa \)-B 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 \( \mu \)g/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. GSK3\( \alpha \)-GST was then used as the substrate for the resulting immunoprecipitated Akt, and the phosphorylated material identified by immunoblot analysis for phosphorylated GSK3\( \alpha \)-GST protein (Fig. 3B). The EGCG treatment

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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 immunoblotting analysis using a tyrosine phosphospecific Her-2/neu antibody (Fig. 4A). A dose-dependent decrease 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.

**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-xB 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-xB 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.
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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 activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature (Lond.), 401: 82–85, 1999.

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