Epigallocatechin-3-gallate Inhibits Activation of HER-2/neu and Downstream Signaling Pathways in Human Head and Neck and Breast Carcinoma Cells

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ABSTRACT

Overexpression of the HER-2/neu receptor (HER-2) is associated with a poor prognosis in patients with breast carcinoma and also in patients with head and neck squamous cell carcinoma (HNSCC). In a previous study on HNSCC cell lines, we found that epigalocatechinen-3-gallate (EGCG), a major biologically active component of green tea, inhibited activation of the epidermal growth factor receptor (EGFR) and thereby inhibited EGFR-related downstream signaling pathways in HNSCC cells. In the present study, we examined the effects of EGCG on activation of the HER-2 receptor in human HNSCC and breast carcinoma cell lines that display constitutive activation of HER-2. Treatment of these cells with 10 or 30 μg of EGCG, respectively, doses that cause 50% inhibition of growth, markedly inhibited the phosphorylation of HER-2 in both cell lines. This was associated with inhibition of Stat3 activation, inhibition of c-fos and cyclin D1 promoter activity, and decreased cellular levels of the cyclin D1 and Bcl-XL proteins. Although these concentrations of EGCG are quite high, we found that concentrations of 0.1–1.0 μg/ml, which are in the range of plasma concentrations after administering a single oral dose of EGCG or a green tea extract, markedly enhanced the sensitivity of both types of cell lines to growth inhibition by Taxol, a drug frequently used in the treatment of breast carcinoma and HNSCC. These results, taken together with previous evidence that EGCG also inhibits activation of the EGFR in carcinoma cells, suggest that EGCG may be useful in treating cases of breast carcinoma and HNSCC in which activation of the EGFR and/or HER-2 plays important roles in tumor survival and growth.

INTRODUCTION

HER-2 (neu/erbB2) is a member of the type 1 RTK superfamily (1). The other three members of this family, EGFR (erbB1), erbB3, and erbB4, are activated by specific ligands, but thus far, no specific ligand for HER-2/neu has been identified (1). Therefore, activation of HER-2 is dependent on dimerization with the EGFR, erbB3, or erbB4 (1). Nevertheless, overexpression of HER-2 is associated with aggressive phenotypes of several types of carcinomas, including increased metastatic ability and resistance to various cancer chemotherapeutic agents (2). Apparently this is because HER-2-containing heterodimers display increased ligand affinity due to a decelerated off-rate, which can potentiate activation of downstream signaling pathways (1). Indeed, 20–30% of breast carcinoma and HNSCC overexpress HER-2, and this overexpression is associated with a poor prognosis in patients with these malignancies (2, 3). Therefore, HER-2 may provide a useful molecular target for therapy. Clinical trials with an anti-HER-2 antibody (Herceptin), used alone or in combination with other agents, are in progress and appear to be promising (2).

In a previous study on HNSCC cell lines (4), we found that EGCG, a major biologically active component of green tea, inhibited activation of the EGFR and thereby inhibited multiple downstream signaling pathways. Thus, EGCG inhibited activation of Stat3, inhibited c-fos and cyclin D1 promoter activity, and decreased cellular levels of the cyclin D1 and Bcl-XL proteins. A recent study indicates that EGCG inhibits the constitutive activation of HER-2 in mouse mammary tumor cell lines (5). In view of these findings, in the present study we examined the effects of EGCG on phosphorylation of HER-2, a marker of the activation of this receptor, in human HNSCC and breast carcinoma cell lines (5). In view of these findings, in the present study we examined the effects of EGCG on phosphorylation of HER-2, a marker of the activation of this receptor, in human HNSCC and breast carcinoma cell lines that display constitutive activation of HER-2. We also examined the effects of EGCG on several molecules that act downstream of activated HER-2-containing heterodimers, including Stat3, c-fos, cyclin D1, and Bcl-XL (6–9). Because HER-2-overexpressing cancer cells are often resistant to treatment with taxanes (2, 10), which are frequently used in the treatment of both HNSCC and breast carcinoma (11, 12), we also examined the combined effects of EGCG and Taxol on inhibition of the proliferation of HNSCC and breast carcinoma cells.

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3 The abbreviations used are: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinoma; EGCG, epigallocatechin-3-gallate; Stat3, signal transducer and activator of transcription 3; p-Stat3, phosphorylated Stat3; TGF-α, transforming growth factor α; β-gal, β-galactosidase.
MATERIALS AND METHODS

Cell Lines, Cell Culture, and Materials. The human HNSCC cell lines YCU-H891 (originally derived from a carcinoma of the hypopharynx) and YCU-N861 (derived from a carcinoma of the nasopharynx) were provided by Dr. M. Tsukuda and have been partially characterized in our previous studies (4, 13). The human breast cancer cell lines BT-474 and MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in a 5% CO₂ atmosphere at 37°C in RPMI 1640 (HNSCC cell lines) or DMEM (breast cancer cell lines) with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). TGF-β1 was obtained from Life Technologies, Inc., and EGCG and Taxol were obtained from Sigma (St. Louis, MO). EGCG was dissolved in water, and Taxol was dissolved in DMSO.

ELISAs for TGF-β1. The levels of TGF-β1 in the conditioned medium were determined by using a TGF-β1 ELISA kit (Oncogene, Boston, MA). In brief, 60–70% confluent cultures were cultured in 35-mm dishes with fresh medium containing 1% calf serum for 24 h. The cell-free conditioned medium was then collected, and the TGF-β1 concentrations were determined according to the manufacturer’s instructions. These concentrations were normalized with respect to the number of cells/plate. Each point represents the average of triplicate wells.

Cell Proliferation Assays. Cell proliferation assays were done essentially as described previously (4, 13), using a PreMix WST-1 Cell Proliferation Assay System (Takara, Tokyo, Japan), according to the manufacturer’s instructions. Cells were treated with the indicated concentrations of drugs for 72 h. Each point represents the average of triplicate wells.

Protein Extraction and Immunoblotting. Total cellular protein was extracted and examined by Western blot analysis with the respective antibodies, as described previously (4, 13). The following primary antibodies were used for the detection of the specific bands: Stat3 (F-2) and phospho-(Y705)-Stat3 (B-7) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); HER-2/neu, phospho-(Y1248)-HER-2/neu, Akt, phospho-(Ser473)-Akt, ERK1/2, and phospho-(Thr 202/Thr 204)-ERK1/2 antibodies (9106S) were obtained from Cell Signaling (Beverly, MA).

Luciferase Reporter Assays. The c-fos luciferase reporter plasmid p-FOS-wt-luc has been described previously (4). The cyclin D1 luciferase reporter plasmid p1745CD1LUC was constructed and provided by Dr. R. Pestell and has also been described previously (4). Assays were done essentially as described previously (4). Briefly, triplicate samples of 1 x 10⁵ cells in 35-mm plates were transfected with Lipofectin (Life Technologies, Inc.). One μg of the reporter plasmid and 10 ng of the pCMV-β-gal plasmid DNA were cotransfected in OptiMEM I medium (Life Technologies, Inc.). After 16 h, the medium was changed to fresh serum-free medium. Half of the cultures were stimulated with 50 ng/ml TGF-β1. The cells were then incubated for 24 h in the presence or absence of EGCG, and luciferase activity was determined with the luciferase assay system (Promega, Madison, WI). β-gal activities were also determined with the β-gal enzyme assay system (Promega).
Luciferase activities were then normalized with respect to β-gal activities.

RESULTS AND DISCUSSION

The HER-2 Protein Is Overexpressed and Constitutively Activated in YCU-H891, BT-474, and MDA-MB-231 Cells. In our initial study, we examined the status of the HER-2 protein by Western blot analyses in two human HNSCC and two breast carcinoma cell lines. Both the total cellular level of HER-2 and its phosphorylated form were expressed at high levels in the YCU-H891 and BT-474 cell lines (Fig. 1A), indicating that in these two cell lines HER-2 is overexpressed and constitutively activated. This was not, however, seen in the YCU-N861 HNSCC or the MDA-MB-231 breast carcinoma cell lines (Fig. 1A). Our findings with BT-474 are consistent with previous evidence that these cells display amplification of the HER-2 gene (14). Therefore, we further analyzed the YCU-H891 and BT-474 cell lines. Because there is evidence that both YCU-H891 cells and BT-474 cells also overexpress the EGFR (4, 14), we assayed the levels of TGF-α, an EGFR-specific ligand, in the 24-h conditioned media of the cells. We found that both cell lines produced relatively high levels of TGF-α. In the YCU-H891 cells, this value was 23 pg/10^6 cells/ml medium, and in the BT-474 cells, it was 63 pg/10^6 cells/ml medium. These results suggest that constitutive activation of HER-2 in these two cell lines is due, at least in part, to autocrine activation of the EGFR by TGF-α (Fig. 1B). The YCU-N861 cells do not produce detectable levels of TGF-α, and the MDA-MB-231 cells produce a moderate amount (10 pg/10^6 cells/ml).

EGCG Inhibits Growth in Both YCU-H891 and BT-474 Cells. To examine the growth-inhibitory effects of EGCG on these two cell lines, the cells were treated with increasing concentrations of EGCG (1–100 µg/ml) for 72 h, and cell proliferation was determined as described in “Materials and Methods” (Fig. 1C). EGCG markedly inhibited the growth of both cell lines. The growth-inhibitory effect was greater in YCU-H891 cells than in BT-474 cells because the respective IC50 concentrations were 10 and 30 µg/ml. In previous studies, we found that the IC50 concentrations for EGCG with YCU-N861 and MDA-MB-231 cells were 10 and 30 µg/ml, respectively (4, 15). In view of this result, it was of interest to examine whether the growth-inhibitory effects of EGCG are associated with inhibition of HER-2 activation in the YCU-H891 and BT-474 cells. The cells were cultured in serum-free medium for 24 h and then grown for an additional 24 h in the absence or presence of the indicated concentrations of EGCG. In addition, in one set of cultures, TGF-α (50 ng/ml) was added to the serum-free medium for 3 h before harvesting the cells. Extracts were then examined by Western blot analysis using antibodies to HER-2 and phospho-HER-2. We found that treatment with IC50 concentrations of EGCG (10 µg/ml for YCU-H891 and 30 µg/ml for BT-474) for 24 h markedly decreased the levels of phosphorylated HER-2 in both the absence and presence of TGF-α (Fig. 2A and B). EGCG did not, however, cause a decrease in the total cellular level of the HER-2 protein in either cell type (Fig. 2). It is of interest that exogenous TGF-α increased the level of phospho-HER-2 in YCU-H891 cells (Fig. 2A), but not in BT-474 cells (Fig. 2B). This may reflect the fact that BT-474 cells produce higher levels of endogenous TGF-α than YCU-H891 cells (Fig. 1B). Therefore, activation of the EGFR/HER-2 heterodimer by TGF-α may be saturated by endogenous TGF-α in BT-474 cells.

EGCG Inhibits Activation of Stat3 in BT-474 Cell Line. Constitutive activation of the oncogenic transcription factor Stat3 (16) frequently occurs in both HNSCC and breast carcinoma, apparently due to overexpression and activation of the EGFR (17, 18). In addition, there is evidence that in malignant human lung epithelial cells TGF-α activation of the EGFR/HER-2 heterodimer is critical for Stat3 activation and that constitutive activation of Stat3, in turn, requires HER-2 kinase activity (6). Therefore, it was of interest to examine the effects of EGCG on Stat3 activation in the BT-474 cells, in which we found that HER-2 is constitutively activated (Fig. 2B). Exponentially growing BT-474 cells were treated with 30 µg/ml EGCG for 48 h, and extracts were prepared and examined by Western blot analysis with antibodies to total Stat3 and p-Stat3 (Fig. 3A). Extracts from nontreated cells contained relatively high levels of total cellular Stat3 and p-Stat3 (Fig. 3A), indicating that Stat3 is constitutively activated in these cells. Treatment with EGCG markedly decreased the levels of p-Stat3 in a time-dependent manner (Fig. 3A). There was also some decrease in the cellular level of the total Stat3 protein at 48 h (Fig. 3A), presumably due to cytotoxicity. Therefore, treatment with EGCG inhibits the constitutive activation of Stat3 in BT-474 cells. These results are consistent with our previous finding that EGCG also markedly inhibits the activation of Stat3 in YCU-H891 cells (4) and with our current finding that EGCG
inhibits the constitutive activation of HER-2 in YCU-H891 cells (Fig. 2A).

EGCG Inhibits c-fos and Cyclin D1 Promoter Activity and Decreases Levels of the Cyclin D1 and Bcl-XL Proteins in BT-474 Cells. There is evidence that c-fos, cyclin D1, and Bcl-XL are downstream targets of the transcription factor Stat3 (7–9). Indeed, in our previous study with YCU-H891 cells (4), we found that inhibition of Stat3 activation by EGCG was associated with inhibition of c-fos and cyclin D1 promoter activity and decreases in cellular levels of the cyclin D1 and Bcl-XL proteins. Therefore, we examined whether EGCG caused similar effects in the BT-474 breast carcinoma cells. When BT-474 cells were treated with 30 μg/ml EGCG, there was a marked decrease in the levels of the cyclin D1 and Bcl-XL proteins at 24 h and a further decrease of the Bcl-XL protein at 48 h (Fig. 3A). We then examined the effects of EGCG on c-fos and cyclin D1 promoter activity in BT-474 cells, using c-fos and cyclin D1 promoter luciferase constructs in transient transfection reporter assays in BT-474 cells. Cells were transfected for 16 h with the respective reporter plasmid and then cultured for 24 h in serum-free medium with or without 50 ng/ml TGF-α, plus 0, 10, or 30 μg/ml EGCG. The exogenous TGF-α did not enhance promoter activity with either the c-fos or cyclin D1 reporters (data not shown), presumably because of the high endogenous production of TGF-α by the BT-474 cells (Fig. 1B). Therefore, Fig. 3B indicates only the results obtained without the addition of TGF-α. EGCG caused a dose-dependent inhibition of the transcriptional activity of both the c-fos and cyclin D1 promoters. For unknown reasons, the inhibitory effects of EGCG were greater with the cyclin D1 promoter than with the c-fos promoter. These results, taken together with the Western blot analysis of cyclin D1 (Fig. 3A), suggest that EGCG inhibits cyclin D1 expression at the level of transcription.

Effects of Combined Treatment with EGCG and Taxol on Growth Inhibition. There is evidence that cancers that overexpress HER-2 are relatively resistant to treatment with taxanes (2), which are important chemotherapeutic agents for both HNSCC and breast carcinoma (11, 12). In view of our evidence that EGCG can inhibit the activation of HER-2 in both types of cancer cells, we examined whether EGCG enhances the growth-inhibitory effects of Taxol (paclitaxel) in YCU-H891 and BT-474 cells. The cells were treated with increasing concentrations of Taxol (0.7–50 ng/ml) alone or plus low concentrations of EGCG (0.1, 1, or 5 μg/ml) that alone do not inhibit cell proliferation (see Fig. 1C) for 72 h, and dose-response curves were generated using cell proliferation assays (Fig. 4A). The subset of these results in which the cells were treated with 1.5 ng/ml Taxol and increasing concentrations of EGCG is displayed in Fig. 4B. In YCU-H891 cells, the addition of only 0.1 μg/ml EGCG significantly enhanced the inhibitory effect of Taxol (Fig. 4, A and B). Thus, treatment with 1.5 ng/ml Taxol alone inhibited the growth of these cells by about 30%, but the addition of 0.1 μg/ml EGCG to 1.5 ng/ml Taxol caused a 60% inhibition of growth (Fig. 4B). These differences were statistically significant (t test, P = 0.0034). The addition of higher doses of EGCG (1.0 or 5.0 μg/ml) to Taxol did not further enhance the growth-inhibitory effects of Taxol (Fig. 4, A and B). In BT-474 cells, the addition of 0.1 μg/ml EGCG to Taxol did not significantly enhance the inhibitory effects of Taxol (Fig. 4, A and B). Thus, both 1.5 ng/ml Taxol alone and 1.5 ng/ml Taxol plus 0.1 μg/ml EGCG caused about 20% inhibition of growth in BT-474 cells (Fig. 4B). However, the addition of 1.0 μg/ml EGCG to 1.5 ng/ml Taxol increased the growth-inhibitory effects of 1.5 ng/ml Taxol by 25% (P = 0.018; Fig. 4B). As with YCU-H891 cells, the addition of 5.0 μg/ml EGCG did not further enhance the growth-inhibitory effects of Taxol (Fig. 4, A and B). Concentrations of 0.1 or 1.0 μg/ml EGCG are in the range of plasma concentrations of EGCG (0.04–0.5 μg/ml) seen in individuals after administering an oral dose of EGCG or a decaffeinated green tea extract (19, 20). Therefore, our results suggest that concentrations of EGCG that can be readily obtained in the serum of humans enhance the growth-inhibitory
effects of Taxol in both HNSCC and breast carcinoma cells. This finding is consistent with our previous finding that EGCG at 0.1 µg/ml markedly enhanced the growth-inhibitory effects of 5-fluorouracil in two HNSCC cell lines (4).

Thus, in the present study, we provide the first evidence that in human breast carcinoma and HNSCC cells, EGCG (10 or 30 µg/ml, respectively) can inhibit phosphorylation and therefore inhibit activation of HER-2. Our findings extend the recent results obtained by Pianetti et al. (5) indicating that EGCG also inhibits phosphorylation of HER-2 in mouse mammary tumors cells. These authors found that this was associated with inhibition of downstream signaling through the phosphatidylinositol 3'-kinase, AKT kinase, and nuclear factor κB pathways. However, we should emphasize that in our studies, we used relatively high concentrations of EGCG (10–30 µg/ml), and the studies by Pianetti et al. (5) used even higher concentrations (i.e., 40–80 µg/ml). However, our cell culture assays were done over a period of 24–48 h, a relatively short period of time. During prolonged administration to patients, EGCG may accumulate in the plasma and/or tumor tissue. Furthermore, as emphasized above, we found that concentrations of EGCG in the range of those found in human plasma after subjects were given a single oral dose of a green tea extract (19, 20) enhanced the growth-inhibitory effects of Taxol. Nevertheless, it remains to be determined whether EGCG can be administered to cancer patients at doses that inhibit activation of the HER-2 receptor in tumor tissue without significant toxicity to normal tissues.

The precise mechanism by which EGCG inhibits phosphorylation of HER-2, as well as other RTKs, remains to be determined. The finding that EGCG inhibits the binding of EGF, platelet-derived growth factor, and fibroblast growth factor to their respective receptors (21, 22) appears to account for the inhibitory effects of EGCG on autophosphorylation of the RTKs, EGFR, platelet-derived growth factor, fibroblast growth factor, and vascular endothelial growth factor receptor 2 in the presence of the respective ligands (21–23). However, a recent study indicates that EGCG can directly inhibit the tyrosine kinase activity of the EGFR in the absence of a ligand for this receptor (24). This finding suggests that EGCG has the potential to act as a kinase inhibitor, probably by binding to the ATP-binding site of RTKs because other types of polyphenolic compounds (namely, genistein and quercetin) inhibit RTKs via this mechanism. Therefore, there are at least two possible explanations for the inhibitory effects of EGCG on HER-2 activation. The first is that EGCG inhibits activation of a heterodimeric partner of HER-2 (for example, the EGFR) and thereby indirectly inhibits HER-2 phosphorylation. The second is that EGCG directly binds to and inhibits the kinase activity of
HER-2. The former mechanism is consistent with the recent finding that in BT-474 cells the EGFR-specific tyrosine kinase inhibitor ZD1839 (Iressa) inhibits the phosphorylation of HER-2 and that the growth-inhibitory effects of ZD1839 were stronger, both in vitro and in vivo, than that of a HER-2-specific antibody (Herceptin). Additional studies are required to determine whether EGCG acts directly on the HER-2 protein or acts indirectly to inhibit the phosphorylation and thereby the activation of HER-2. Nevertheless, the findings of the present study suggest that EGCG provides a natural product that may be useful when used in combination with other antitumor agents in the treatment of cases of HNSCC and breast carcinoma in which activation of both EGFR and HER-2 plays critical roles in tumor survival and growth.

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