(−)-Epigallocatechin Gallate and Polyphenon E Inhibit Growth and Activation of the Epidermal Growth Factor Receptor and Human Epidermal Growth Factor Receptor-2 Signaling Pathways in Human Colon Cancer Cells

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Abstract

Purpose: (−)-Epigallocatechin gallate (EGCG) inhibits activation of the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2) and multiple downstream signaling pathways in cancer cell lines. In this study we compared the cellular and molecular effects of EGCG with a well-standardized decaffeinated green tea catechin mixture Polyphenon E (Poly E) on human colon cancer cell lines.

Experimental Design and Results: Both EGCG and Poly E preferentially inhibited growth of the Caco2, HCT116, HT29, SW480, and SW837 colon cancer cells when compared with the FHC normal human fetal colon cell line. The EGFR and HER2 proteins were overexpressed and constitutively activated in all of the colon cancer cell lines when compared with the FHC cell line. Treatment of HT29 cells with EGCG or Poly E caused an increase of cells in G_1 and induced apoptosis. Both EGCG and Poly E caused a decrease in the phosphorylated forms of EGFR and HER2 proteins, and subsequently caused a decrease in the phosphorylated forms of the extracellular signal-regulated kinase and Akt proteins. Similar effects of these compounds were seen when the cells were stimulated with transforming growth factor α. Reporter assays indicated that both EGCG and Poly E inhibited the transcriptional activity of the activator protein 1 (AP-1), c-*fos*, nuclear factor κB, and cyclin D1 promoters. The combination of only 1 μg/mL of epicatechin plus 10 μg/mL of EGCG displayed synergistic effects on growth inhibition and induction of apoptosis. Furthermore, when treatment was prolonged for 96 hours, 1 μg/mL of EGCG or Poly E was sufficient to inhibit growth, reduce activation of EGFR and HER2, and induce apoptosis.

Conclusion: Our findings suggest that EGCG or Poly E may be useful in the chemoprevention and/or treatment of colon cancer. Poly E contains about 60% EGCG, yet pure EGCG and Poly E had similar potencies (expressed as μg/mL). Poly E may be preferable because it is easier to prepare and this mixture of catechins may exert synergistic effects.

Epidermal growth factor receptor (EGFR; erbB1) and human epidermal growth factor receptor-2 (HER2) and (neu/erbB2) belong to subclass I of the receptor tyrosine kinase superfamily (1). Three members of this family, EGFR, HER3 (erbB3), and HER4 (erbB4), are activated by specific ligands, but thus far, no specific ligand for HER2 has been identified (1). Ligand binding results in receptor homo- and heterodimerization leading to phosphorylation of tyrosine residues, activation of downstream signaling pathways, and expression of genes that enhance cell proliferation (1). HER2 is the preferred heterodimerization partner for the EGFR, HER3, and HER4 (1). Abnormalities in the expression of growth factors and their receptors play a critical role in the development of human malignancies including colorectal cancer. Thus, human colorectal carcinoma often displays overexpression of EGFR and one of its ligand, transforming growth factor α (TGF-α; refs. 2, 3). This results in constitutive activation of the tyrosine kinase activity of the EGFR and related downstream signaling pathways. This process can occur even in the early stages of human colorectal carcinogenesis (4–6). HER2 is also frequently overexpressed in colorectal carcinoma when compared with normal colonic mucosa, and the extent of overexpression seems to correlate with increasing disease stage and poorer patient survival (7). Therefore, therapies that target the EGFR and/or HER2 receptor may be effective in the chemoprevention and/or therapy of colorectal cancer (8).

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Tea is one of the most popular beverages in the world. Epidemiologic studies suggest that the consumption of tea, especially green tea, is linked to a decreased incidence of various cancers including colorectal cancer (9–11). Over 40 experimental studies in rodents have shown that green tea or its constituents can either inhibit carcinogenesis or the growth of established cancers at various organ sites including the colon (12). Green tea contains several polyphenolic compounds including the catechins (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). EGCG is one of the major constituents of green tea (9, 10), and it seems to be the most potent compound in tea with respect to inhibiting cell proliferation and inducing apoptosis in cancer cells (13, 14). Liang et al. (15) discovered that EGCG binds to and inhibits the tyrosine kinase activity of EGFR in human A431 epidermoid carcinoma cells. We recently reported that EGCG inhibits activation of the EGFR, and also HER2, and multiple downstream signaling pathways in human head and neck squamous cell carcinoma (HNSCC) and breast cancer cell lines (16, 17). Thus, EGCG inhibits activation of extracellular signal-regulated kinase (ERK), inhibits basal and TGF-α–stimulated c-*fos* and cyclin D1 promoter activity, and causes a decrease in cellular levels of the cyclin D1 and Bcl-x_L proteins (16, 17). Other investigators found that EGCG also inhibits HER2 receptor tyrosine phosphorylation in a mouse mammary tumor cell line and this was associated with inhibition of phosphatidylinositol 3 (PI3)-kinase, Akt kinase, and nuclear factor κB (NF-κB) signaling pathways (18). In the present study, we examined the effects of EGCG on activation of EGFR and HER2 in the HT29 human colon cancer cell line that overexpresses both of these receptors, and we also examined the effects of EGCG on related downstream signaling pathways. In parallel with our studies on EGCG, we also examined in HT29 cells the effects of Polyphenon E (Poly E), a standardized and well-characterized decaffeinated extract of green tea that contains 60% EGCG and lesser amounts of the catechins EGC, ECG, EC, and gallocatechin-3-gallate (GCG). Poly E is of interest because it is readily prepared and its components may exert synergistic or additive effects with respect to antitumor activity (19).

Materials and Methods

Chemicals. EGCG and Poly E were provided by the National Cancer Institute (Bethesda, MD). Poly E contains about 60% EGCG, 7% EC, 12% EGC, 1% ECG, and 2% GCG, respectively (20). Therefore, 20 μg/mL of Poly E contain about 12 μg/mL of EGCG and 1.4 μg/mL of Poly E.

Cell lines and cell culture. The Caco2, HCT116, HT29, SW480, and SW837 human colorectal cancer cell lines and the FHC normal human fetal colon cell line were obtained from American Type Culture Collection (Manassas, VA). All of the cell lines were maintained in DF10 medium containing DMEM (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (Invitrogen). The FHC cell line was originally established from the colon of a fetus at 13 weeks of gestation and has an epithelial morphology. Cells were cultured in an incubator with humidified air at 37°C with 5% CO_2. As an untreated solvent control, cells were treated with DMSO (Sigma Chemical Co., St. Louis, MO) at a final concentration of <0.1%.

Cell viability assays. Cell viability assays were done essentially as previously described (21) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit I (Roche Diagnostics Co., Indianapolis, IN), according to the instructions of the manufacturer. The five colorectal cancer cell lines and the FHC cell line were plated onto 96-well plates (3×10^3 cells/well). Twenty-four hours later, the cells were treated with the indicated concentrations (0 to 50 μg/mL) of EGCG or Poly E for 48 hours in DF10 medium. The medium and drugs were not changed during this time period. All assays were done in triplicate. To examine the effects of TGF-α on the growth of the HT29 and FHC cells, the cells were treated with the indicated concentrations (0 to 50 ng/mL) of exogenous TGF-α for 48 hours in DMEM medium in the absence of serum, and cell viability assays were then done using the MTT system.

(−)-Epigallocatechin gallate and epicatechin combination assays. Three-thousand HT29 cells were plated onto 96-well plates. Twenty-four hours later, EGCG and EC were added alone or in combination at the indicated concentrations to each well, and cells were incubated for 48 hours in DF10 medium. Cell viability assays were then done using the MTT system as described above. To determine whether the combined effects of EGCG plus EC were synergistic, the combination index isobologram was used to analyze the data obtained (22, 23).

Cell proliferation assays. One hundred thousand HT29 cells were seeded in multiple 35-mm diameter dishes in DF10 medium, and 24 hours later they were treated with the indicated concentrations (1 or 20 μg/mL) of EGCG or Poly E for 96 hours. The medium and drugs were not changed during this time period. As a control, the cells were treated with 0.1% DMSO. Every 24 hours up to 96 hours, replicate plates of cells were washed with PBS and the attached cells were collected by trypsinization. The numbers of cells in replica plates were then determined using a Coulter Counter (Beckman Coulter Co., Fullerton, CA) as previously described (24).

Protein extraction and Western blot analysis. Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis as previously described (21, 23). The primary antibodies used for detection of the respective protein bands were also previously described (16, 17, 25). An antibody to actin was used as a loading control. Each membrane was developed using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The intensities of protein bands were quantified using NIH Image software version 1.62, as previously described (23).

Cell cycle assays. HT29 cells were seeded into 6-cm diameter dishes (5×10^5 cells/dish) in DF10 medium and cultured for 24 hours to allow for cell attachment. The cells were then treated with 20 μg/mL EGCG, 20 μg/mL Poly E, or 0.1% DMSO for the indicated times (0 to 48 hours). The cells were stained with propidium iodide (Sigma) and were then analyzed for DNA histograms and cell cycle phase distribution by flow cytometer using a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ); data were analyzed using the CELL Quest computer program (Becton Dickinson) as previously described (21, 23).

DNA fragmentation assays. To quantify the induction of apoptosis, a DNA fragmentation assay was done using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics), which detects the presence of histone-associated DNA fragmentation in the cell cytosol according to the instructions of the manufacturer and as previously described (23, 24). HT29 cells were seeded into 6-cm diameter dishes (5×10^5 cells/dish) in DF10 medium, and 24 hours later the cells were then treated with the indicated concentrations of chemicals for the indicated times. The extent of DNA fragmentation at time 0 hour was set to 1 and increases expressed as fold activation.

Caspase activity assays. Caspase-3 and -9 activity assays were done using a fluorometric system as previously described (23, 26). HT29 cells (5×10^5 cells/6-cm diameter dish) were plated and 24 hours later the cells were treated with 20 g/mL EGCG, 20 g/mL Poly E, or 0.1% DMSO for 24 or 48 hours. Total cell extracts (10 μg of total protein) were then incubated with 2 μg of the fluorogenic peptide substrates Ac-DEVD-AFC (PharMingen, San Diego, CA) or Ac-LEHD-AFC (EMD Biosciences Inc., San Diego, CA), the fluorogenic substrates for caspase-3 and -9, respectively, in a caspase buffer. Caspase activities were calculated by the release of AFC, which was measured with a Gemini Fluoro/Luminometer (Molecular Devices Co., Sunnyvale, CA). The caspase activities found at 0 hour were set to 1, and changes were shown as fold activation.

AP-1, c-fos, nuclear factor κB, and cyclin D1 reporter assays. Reporter assays were done as previously described (16, 17, 25). The pAP-1-luciferase reporter plasmid was kindly provided by Dr. J.H. Pierce (National Cancer Institute, Bethesda, MD; ref. 27). The cyclin D1 promoter luciferase reporter plasmid −1745CD1LUC was provided by Dr. R.G. Pestell (Georgetown University, Washington, DC; ref. 28). The c-*fos* promoter luciferase reporter plasmid pFos-wt-luc and the (NF-κB) promoter luciferase reporter plasmid pNF-κB-Luc were previously described (16, 17, 25). HT29 cells (2×10^5 cells/35-mm diameter dish) were transfected with 1 μg of DNA of the indicated luciferase reporter plasmid using a Lipofectin reagent (Invitrogen) in opti-MEM I medium (Invitrogen) for 24 hours. The cells were then treated for 24 hours with the indicated concentrations (10 or 20 μg/mL) of EGCG or Poly E in serum minus DMEM medium which contained or lacked 50 ng/mL of TGF-α, and extracts were then prepared. Luciferase activities were determined using a luciferase assay system (Program Co., Madison, WI). In all of these reporter assays the cells were also cotransfected with a CMV-β-galactosidase reporter. Luciferase activities were normalized with respect to the β-galactosidase activities to correct for differences in transfection efficiency as previously described (23, 24).

Statistical analysis. Statistical analyses of the TGF-α stimulation assays, DNA fragmentation assays, caspase assays, and reporter assays were analyzed by Student's *t* test. The results were considered statistically significant if the *P* value was less than 0.05.

Results

(−)-Epigallocatechin gallate and Polyphenon E preferentially inhibit the growth of human colon cancer cells when compared with FHC normal human fetal colon cells. In our initial study we examined the growth inhibitory effects of EGCG and Poly E on five human colon cancer cell lines and on the FHC normal human fetal colon cell line using MTT assays (Fig. 1; Table 1). We found that EGCG and Poly E inhibited growth of the Caco2, HCT116, HT29, and SW837 colon cancer cell lines with an IC_{50} value of about 20 μg/mL (equivalent to about 30 to 40 μmol/L EGCG). The SW480 colon cancer cells were more resistant to EGCG and Poly E because the IC_{50} value with both materials was about 37 μg/mL. The FHC human fetal colon cells were the most resistant because the IC_{50} value with both compounds was about 50 μg/mL. These results suggest that EGCG and Poly E preferentially inhibit the growth of human colorectal cancer cells. In addition, although Poly E contains only about 60% EGCG, it was similar in potency to that of pure EGCG when compared on the basis of μg/mL (Fig. 1; Table 1).

The epidermal growth factor receptor and HER2 proteins are overexpressed and constitutively activated in colon cancer cell lines. Because of the frequent abnormalities in expression and function of the EGFR family of tyrosine kinases in the growth of cancer cells (1, 3), and our previous studies indicating that EGCG inhibits activation of EGFR and HER2 in human HNSCC and breast cancer cells (16, 17), we examined by Western blot analysis whether the EGFR and HER2 proteins are overexpressed and activated in this series of colon cancer cell lines and in the FHC cell line (Fig. 2*A* and *B*). Although there were no major differences in expression levels of EGFR protein among these cell lines, the level of p-EGFR protein was increased 3.2- to 4.2-fold in the colon cancer cell lines compared with its level in the FHC cell line (Fig. 2*A*). The level of HER2 protein was markedly increased in all of the colon cancer cell lines when

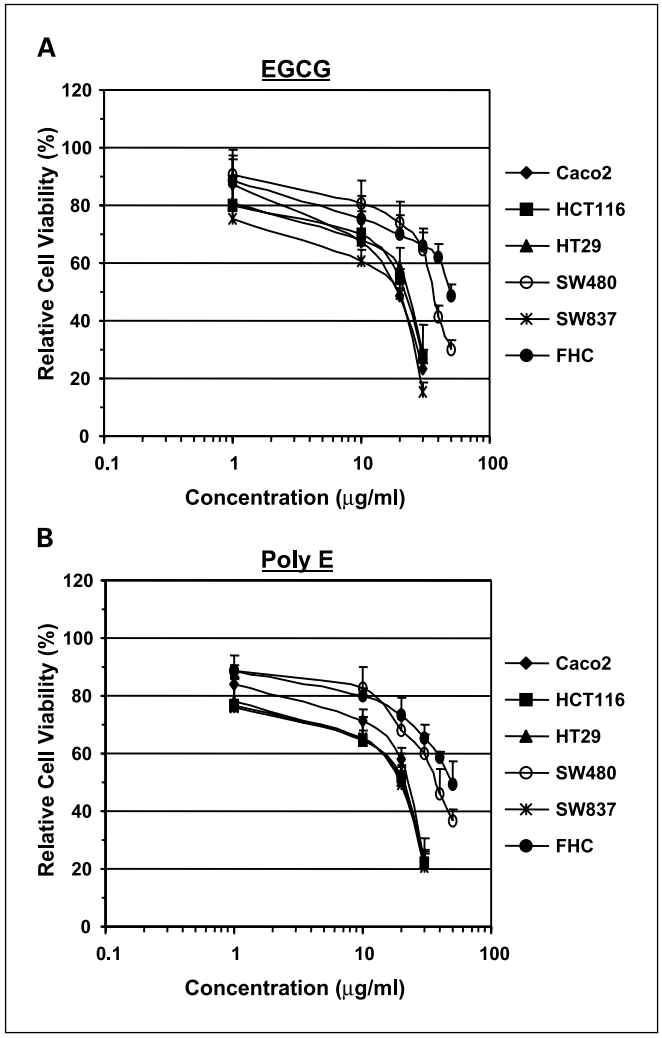

Fig. 1. Inhibition of cell growth by EGCG (A) or Poly E (B) in human colon cancer cell lines and in FHC fetal colon cells. Results are expressed as percentage of growth with 100% representing control cells treated with DMSO alone. *Bars,* SD of triplicate assays.

compared with the FHC cell line especially in the HT29 and SW837 cell lines which displayed an increase of 8.4-fold. There was also an increase in the level of p-HER2 protein in the colon cancer cell lines, except for the SW480 cell line (Fig. 2*B*).

The lower levels of expression of HER2 and p-HER2 proteins in the SW480 cells are of interest because these cells were more resistant to growth inhibition by EGCG and Poly E than the other colon cancer cell lines (Fig. 1; Table 1), although other factors may play a role in the relative resistance of SW480 cells. These results suggest that inhibition of the activation of EGFR, and especially inhibition of HER2 activation, may play an important role in the inhibition of growth of colon cancer cells by EGCG and Poly E. Although no direct ligand for HER2 has yet been discovered, there is increasing evidence that HER2 is the preferred heterodimerization partner for the other members of the erbB family of receptor tyrosine kinases (1, 29, 30). Therefore, HER2 may play a critical role in the potentiation of erbB receptor signaling (1, 29, 30). Thus, agents like EGCG and Poly E that target both the EGFR and HER2 may provide

Table 1. Effects of EGCG and Poly E on the growth of human colon cancer cells (Caco2, HCT116, HT29, SW480, and SW837) and normal human fetal colon cells (FHC)

	IC_{50}*			
	EGCG		Poly E	
Cell lines	μg/mL	μmol/L	μg/mL	μmol/L†
Caco2	19.2	42.2	22.3	29.4
HCT116	21.7	47.7	20.6	27.2
HT29	22.8	50.2	20.1	26.5
SW480	36.4	80.1	37.0	48.8
SW837	19.6	43.1	19.6	25.9
FHC	48.8	107.4	49.4	65.2

*IC_{50} equals concentration that caused 50% inhibition of growth based on data shown in Fig. 1.
†Expressed as concentration of EGCG.

an effective strategy for inhibiting the growth of colon cancer cells that display overexpression and activation of both proteins.

Transforming growth factor α stimulates the growth of HT29 colon cancer cells but not the growth of FHC normal fetal colon cells. TGF-α is one of the most potent of the several ligands that bind to and activate the EGFR (1). Therefore, we examined whether TGF-α preferentially stimulates the growth of colon cancer cells compared with normal colon epithelial cells (Fig. 2*C*). Because the HT29 cell line displays overexpression and constitutive activation of both the EGFR and HER2 proteins (Fig. 2*A* and *B*), these studies were done with this cell line. When HT29 cells were treated with concentrations of 1 to 50 ng/mL of TGF-α, we found a significant and concentration-dependent stimulation of cell growth; 50 ng/mL caused a 3-fold stimulation of growth. In contrast, no stimulation of growth was observed when FHC cells were treated with similar concentrations of TGF-α (Fig. 2*C*). Moreover, treatment of FHC cells with 50 ng/mL TGF-α for 24 hours did not cause a substantial increase in the levels of p-EGFR or p-HER2 proteins (Fig. 2*D*), nor did it cause an increase in the levels of p-ERK or p-Akt proteins (data not shown) in FHC cells. However, as discussed below, similar treatment with TGF-α caused a marked increase in the levels of p-EGFR, p-HER2, p-ERK, and p-Akt proteins in HT29 cells.

These results provide evidence that the extent of activation of EGFR and its heterodimeric partner HER2 can play an important role in the growth of colon cancer cells, thus indicating that the EGFR and HER2 signaling pathways may be useful targets for inhibiting the growth of colon cancer cells. The very low levels of expression of HER2 and p-HER2 in FHC cells (Fig. 2*B*) may explain their lack of response to growth stimulation by TGF-α (Fig. 2*C*) because as a heterodimeric partner of EGFR, HER2 is a critical regulator in the potentiation of erbB receptor signaling (1, 29, 30). Taken together, our findings with EGCG and Poly E in HT29 cells (Fig. 1; Table 1) are consistent with the finding that ZD1839 (Iressa), a selective EGFR-tyrosine kinase inhibitor, showed potent growth inhibition in human colon cancer cell lines both *in vitro* and in xenografts in mice (31).

(−)-Epigallocatechin gallate and Polyphenon E cause HT29 cells to arrest in the G_1 phase of the cell cycle, induce apoptosis, and lead to activation of caspase-3 and -9 in HT29 cells. EGCG can induce G_1 phase arrest of the cell cycle and induce apoptosis in human HNSCC cells (16). To determine whether the growth inhibition that we observed in colon cancer cell lines was associated with specific changes in cell cycle distribution, cell cycle analysis was done using DNA flow cytometry (Fig. 3*A*). When HT29 cells were treated with 20 μg/mL EGCG or Poly E for 48 hours, the percentage of cells in G_1 increased by 26.4% and 24.2%, respectively, and with both agents this was associated with a concomitant decrease of cells in the S and G_2-M phases of cell cycle. No significant changes in cell cycle distribution were observed in the DMSO-treated control cells during this time course (Fig. 3*A*). Western blot analysis indicated that the expression levels of the cyclin D1 and Bcl-x_L proteins displayed a decrease when the cells were treated with 20 μg/mL of either EGCG or Poly E for 24 hours (Fig. 3*B*). Furthermore, 20 μg/mL of EGCG or Poly E caused induction of apoptosis because after treatment for 48 hours the DNA fragmentation ratio increased by 3.5- to 4.0-fold (Fig. 3*C*). Treatment with EGCG or Poly E also caused a significant increase in both caspase-3 and -9 activities within 24 hours. This effect was especially striking with caspase-3, which displayed about a 5.0-fold increase when the cells were treated with EGCG or Poly E for 48 hours (Fig. 3*D*). Therefore, both EGCG and Poly E seem to be equally effective in causing a G_1 phase arrest of the cell cycle and in inducing apoptosis in HT29 cells.

(−)-Epigallocatechin gallate and Polyphenon E cause a decrease in the phosphorylated forms of the epidermal growth factor receptor, HER2, extracellular signal-regulated kinase, and Akt proteins in HT29 cells. Because EGCG can inhibit the activation of EGFR and HER2 and their downstream signaling pathways in HNSCC and breast cancer cells (16, 17), we then examined whether EGCG and Poly E also inhibit activation of the EGFR and HER2 and downstream signaling pathways in colon cancer cells (Fig. 4). When we examined time-dependent effects of EGCG and Poly E in HT29 cells (Fig. 4*A*), there was a marked decrease in the levels of p-EGFR and p-HER2 proteins within 6 hours after treatment with either agent. The expression levels of p-Akt and p-ERK proteins also displayed a marked decrease at 6 and 12 hours, respectively, after addition of either agent. During this period, there was no significant change in the total level of the respective proteins (Fig. 4*A*). We then examined whether EGCG and Poly E substantially inhibit TGF-α–stimulated EGFR and HER2 activation and TGF-α activation of downstream signaling pathways (Fig. 4*B*). Treatment with 50 ng/mL TGF-α caused an increase in the levels of p-EGFR, p-HER2, p-ERK, and p-Akt proteins in HT29 cells. Both in the presence and absence of TGF-α stimulation, 20 μg/mL of EGCG or Poly E caused a marked decrease in the levels of p-EGFR, p-HER2, p-ERK, and p-Akt proteins without having a significant effect on the total abundance of the respective proteins (Fig. 4*B*). Thus, these compounds can overcome the stimulatory effects of TGF-α on these pathways.

(−)-Epigallocatechin gallate and Polyphenon E inhibit the transcriptional activity of the AP-1, c-fos, nuclear factor κB, and cyclin D1 promoters. Aberrant activation of the (AP-1) and NF-κB transcription factors often plays an important role in the

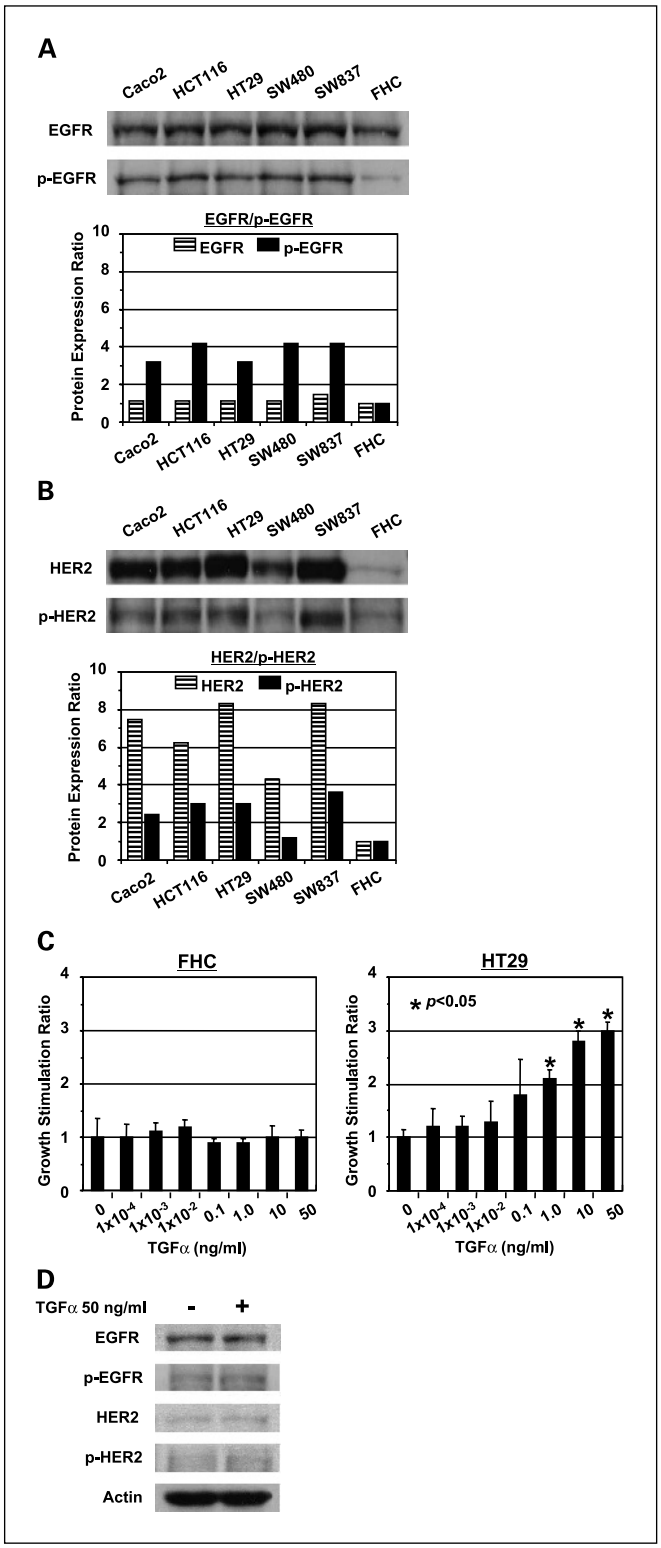

Fig. 2. The expression levels of EGFR, p-EGFR, HER2, and p-HER2 proteins in colon cancer cell lines and the FHC fetal colon cell line. Total protein extracts were prepared from 70% confluent cell cultures of the indicated cell lines and equivalent amounts of protein (60 μg/lane) were examined by Western blot analysis for EGFR and p-EGFR (A) or HER2 and p-HER2 (B), using the appropriate antibodies, as described in Materials and Methods. The results were quantitated by densitometry and these values are displayed in the lower panels, respectively. Repeat Western blots gave similar results. C, effects of TGF-α on the growth of HT29 colon cancer and FHC fetal colon cells. The cells were grown in the presence of the indicated concentration of TGF-α in serum-free DMEM medium and growth measured by MTT assays. Growth stimulation ratio is expressed as fold increase versus the TGF-α – untreated cells. *Bars,* SD of triplicate assays. D, effects of TGF-α on the level of the phosphorylated forms of EGFR and HER2 proteins in FHC cells. The FHC cells were stimulated with 50 ng/mL TGF-α for 24 hours and cell extracts were then examined by Western blot analysis using the respective antibodies. An antibody to actin served as a loading control. Similar results were obtained in a repeat experiment.

development of cancer (32, 33). Activation of the mitogen-activated protein kinase pathway leads to activation of AP-1 (34) and activation of the Akt pathway leads to activation of NF-κB (35). The cyclin D1 promoter contains binding sites for both AP-1 and NF-κB (28, 36). In view of our finding that EGCG and Poly E inhibited activation of both the ERK and Akt proteins (Fig. 4), we then examined the effects of these chemicals on the transcriptional activity of the AP-1, c-*fos*, NF-κB, and cyclin D1 promoters using transient transfection luciferase reporter assays. As shown in Fig. 5, in the presence or absence of TGF-α, treatment with EGCG or Poly E caused a dose-dependent inhibition of AP-1 (*A*), c-*fos* (*B*), NF-κB (*C*),

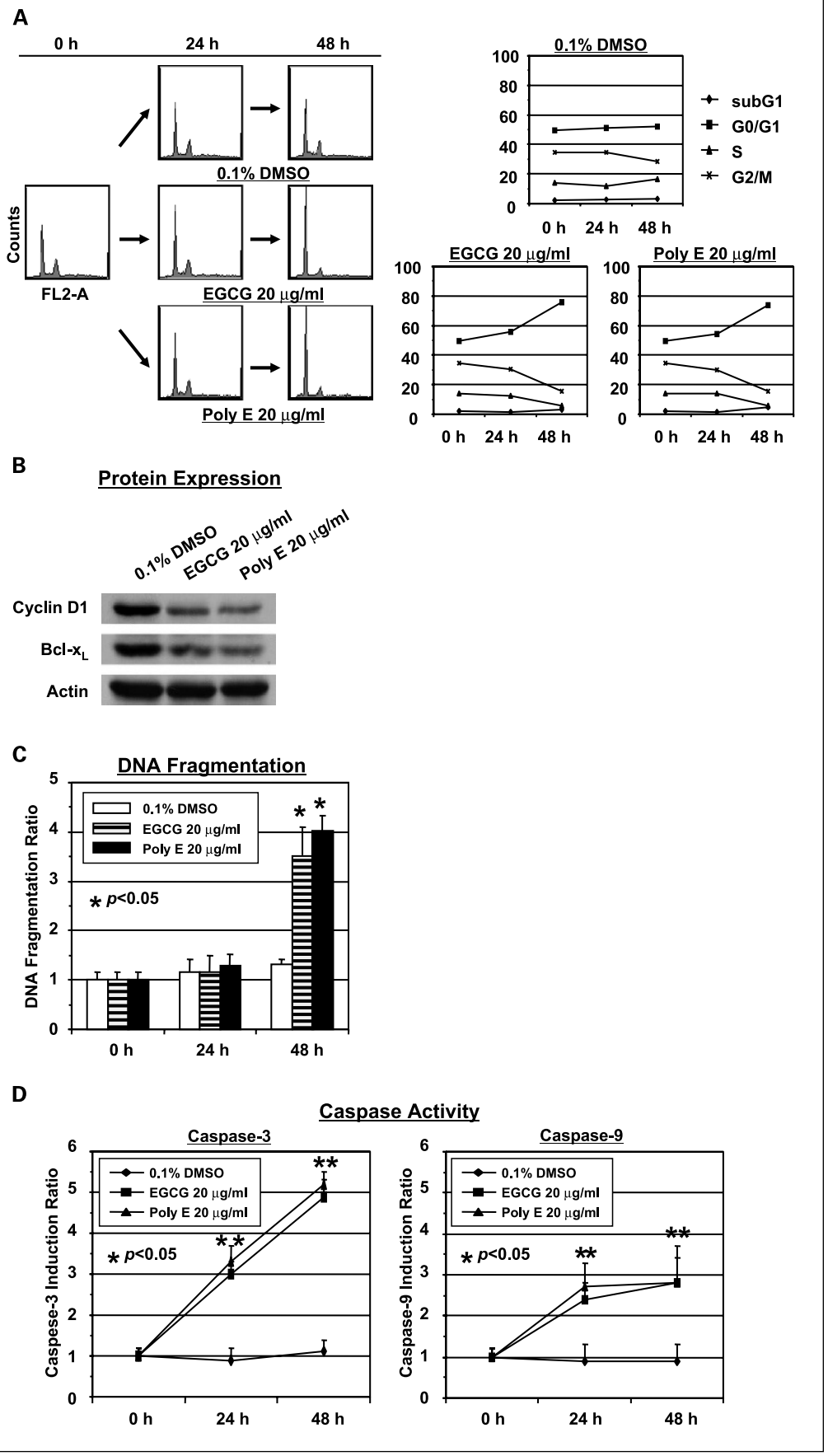

Fig. 3. Effects of EGCG and Poly E on cell cycle progression, induction of apoptosis, and caspase activities in HT29 cells. The cells were treated with 20 μg/mL EGCG, 20 μg/mL Poly E, or 0.1% DMSO for the indicated times, and then analyzed by DNA flow cytometry (A); cell extracts were examined for DNA fragmentation (C) and caspase-3 and -9 activity (D). A, histograms of the cell cycle analysis (*left*); distribution of cells in the sub-G_1, G_0/G_1, S, and G_2-M phases of the cell cycle are calculated and plotted (*right*). C and D, data on DNA fragmentation and caspase activity, respectively. Each asterisk indicates a significant difference ($P < 0.05$) between the control untreated cells and the EGCG- or Poly E-treated cells. *Bars,* SD of triplicate assays. B, levels of cyclin D1 and Bcl-x_L proteins in HT29 cells. The HT29 cells were treated with 20 μg/mL EGCG or Poly E for 24 hours, and cell extracts were then examined by Western blot analysis using the respective antibodies. An antibody to actin served as a loading control. Similar results were obtained in a repeat experiment.

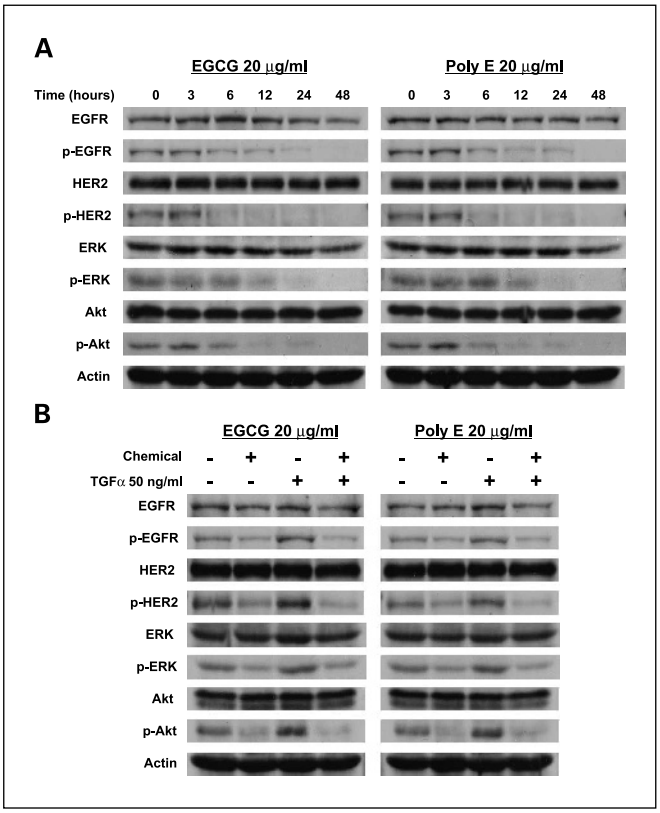

Fig. 4. Effects of EGCG and Poly E on activation of EGFR and HER2 and on related downstream signaling pathways. A, HT29 cells were treated with 20 μg/mL EGCG or Poly E for the indicated times (0, 3, 6, 12, 24, and 48 hours), and cell extracts were then examined by Western blot analysis using the respective antibodies. B, as indicated, HT29 cells were treated with 20 μg/mL EGCG or Poly E for 24 hours in the presence or absence of 50 ng/mL TGF-α. Cell extracts were then examined by Western blot analysis using the respective antibodies. An antibody to actin served as a loading control. Similar results were obtained in a repeat experiment.

and cyclin D1 (*D*) luciferase reporter activities. This inhibition was most apparent with respect to c-*fos* promoter activity. Thus, when HT29 cells were treated with 10 μg/mL EGCG or Poly E in the presence of TGF-α, c-*fos* luciferase reporter activity was inhibited by about 60% (Fig. 5*B*). This effect is consistent with evidence that EGCG inhibits AP-1 activation and cell transformation in JB6 cells (37) and also inhibits Ras-activated AP-1 activity in H-*ras* – transformed JB6 cells (38).

Our finding that EGCG inhibits c-*fos*, NF-κB, and cyclin D1 transcriptional activities in HT29 colon cancer cells is consistent with our previous studies on the effects of EGCG in human HNSCC and breast cancer cells (16, 17, 25). The inhibition of cyclin D1 promoter activity (Fig. 5*D*) suggests that EGCG and Poly E inhibit cyclin D1 expression (Fig. 3*B*) at the level of transcription, thus causing a G_1 arrest of the cell cycle (Fig. 3*A*). Ahmad et al. (39) found that EGCG caused inhibition of cell growth, G_1 arrest of the cell cycle, and induction of apoptosis in human epidermoid carcinoma cells, but did not cause these effects in normal human epidermal keratinocytes. The authors suggested that this was due to the fact that EGCG inhibited activation of NF-κB in carcinoma but not in normal cells (39), but other factors may also play a role. In studies on murine mammary tumor cells, EGCG also inhibited the PI3-kinase/Akt/NF-κB pathway, and the authors suggested that this was mediated by inhibition of HER2 receptor tyrosine phosphorylation (18).

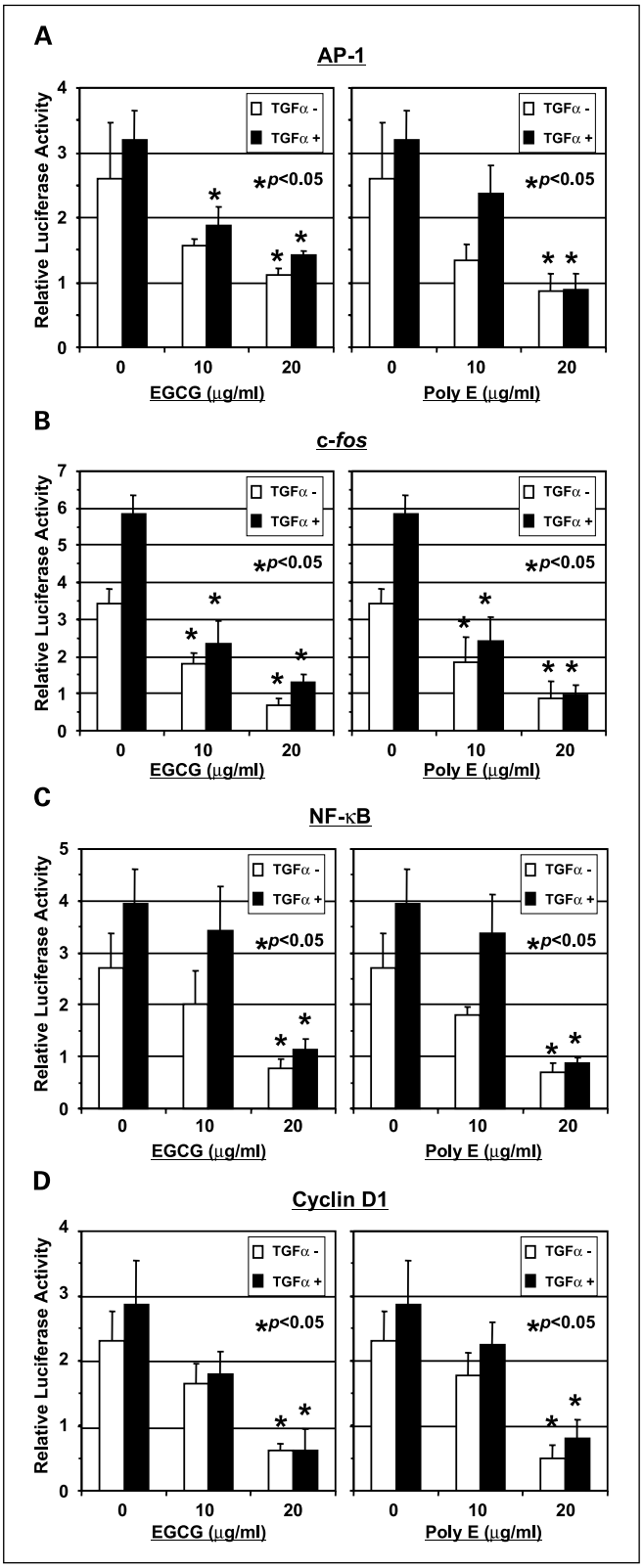

Fig. 5. Effects of EGCG and Poly E on transcriptional activity of the AP-1 (A), c-*fos* (B), NF-κB (C), and cyclin D1 (D) promoters in HT29 cells. Transient transfection reporter assays were done with the indicated reporter in the presence of the indicated concentrations of DMSO, EGCG, or Poly E in the absence or presence of 50 ng/mL TGF-α, as indicated. Relative luciferase activity was then determined after 24 hours. Asterisks indicate a significant difference ($P < 0.05$) between control untreated cells and EGCG- or Poly E -treated cells. *Bars,* SD of triplicate assays. For additional details, see Materials and Methods.

(−)-Epigallocatechin gallate plus epicatechin causes synergistic inhibition of growth and induction of apoptosis in HT29 cells. Poly E contains five different catechins, EC, EGC, ECG, GCG, and EGCG. EGCG constitutes only 60% of this mixture. To clarify why Poly E caused growth inhibitory effects on colon cancer cells equivalent to that of EGCG alone (Fig. 1; Table 1), we examined the growth inhibitory effects of a combination of EGCG plus EC on HT29 cells (Fig. 6*A* and *B*). We also examined whether the combination of EGCG plus EC acts synergistically to induce apoptosis in these cells (Fig. 6*C*). We specifically chose EC because a previous study indicated that it can significantly enhance inhibition of growth of PC-9 lung cancer cells by EGCG (19). When tested alone, EC inhibited growth of HT29 cells with an IC_{50} value of about 80 μg/mL (276 μmol/L; Fig. 6*A*). Because in HT29 cells EGCG has an IC_{50} value of about 23 μg/mL (50 μmol/L; Fig. 1; Table 1), on a molar basis EC is a very weak inhibitor of growth of these cells. We then examined the effects on growth of combining various concentrations of these two catechins (Fig. 6*A*). To determine whether any of these combinations exerted antagonism, additively, or synergy, these data were analyzed by the isobologram method (refs. 22, 23; Fig. 6*B*; Table 2). We found that the combination of as little as 1 μg/mL of EC with 10 or 20 μg/mL of EGCG produced combination indices of 0.88 and 0.71, respectively (Fig. 6*B*). According to the isobologram analysis method (22, 23), these indices are indicative of "slight" and "moderate" synergism, respectively (Table 2). This synergistic effect was not seen with 0.1 or 1 μg/mL of EGCG (Table 2), indicating that the synergistic effect seems to require a minimum concentration of EGCG and/or optimum ratio of EC to EGCG (see Discussion).

We also examined the effects of combining increasing concentrations of EC (1 to 100 μg/mL) with 0 or 10 μg/mL of EGCG on induction of apoptosis using the DNA fragmentation assay (Fig. 6*C*). When treated alone, at 48 hours only the 100 μg/mL dose of EC caused a significant increase in apoptosis; and when tested alone, the 10 μg/mL dose of EGCG caused a slight but not statistically significant increase in apoptosis. However, the combination of as little as 1 μg/mL of EC plus 10 μg/mL of EGCG caused a 2- to 3-fold and significant

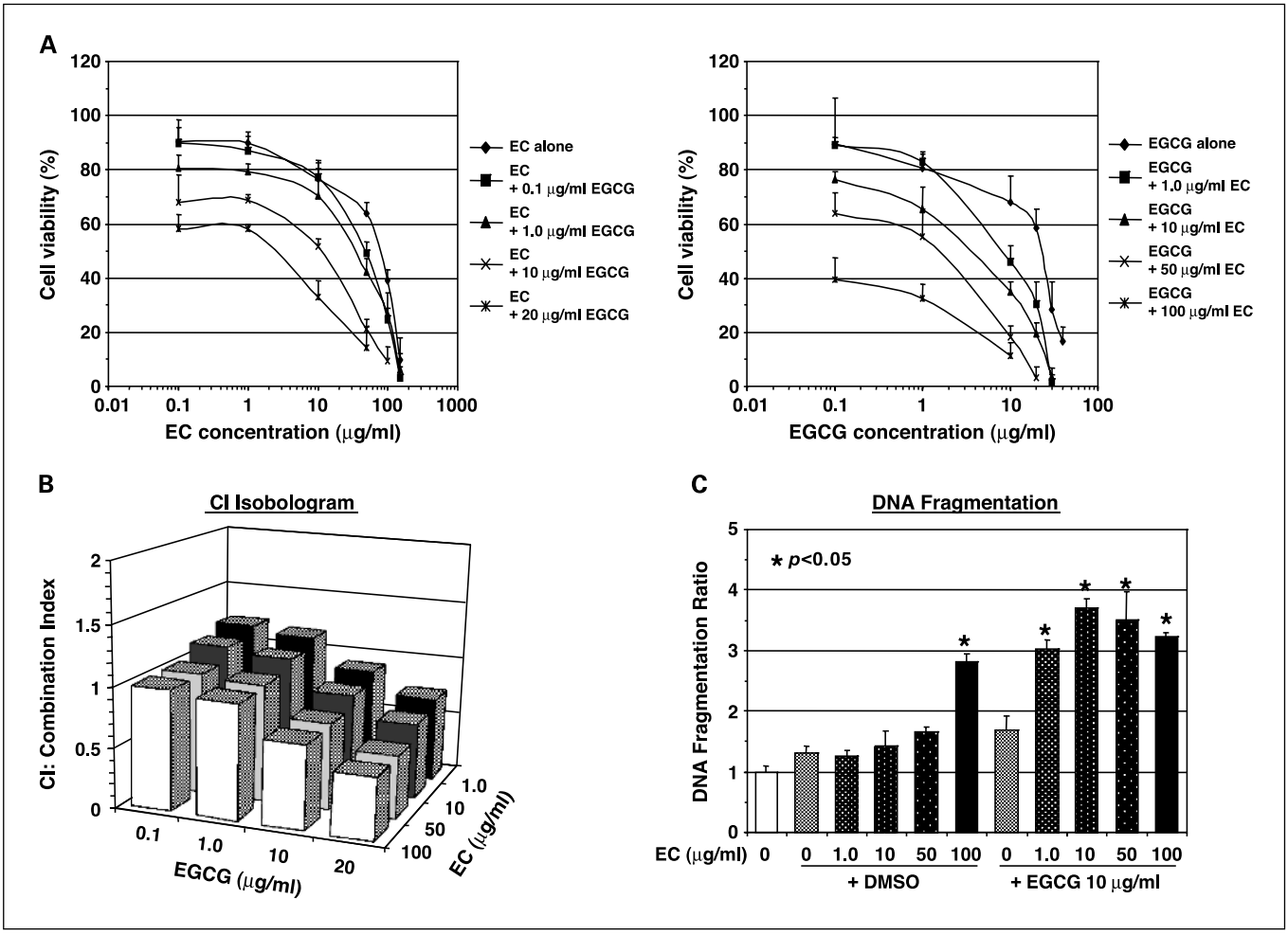

Fig. 6. Inhibition of growth and induction of apoptosis in HT29 cells by EC alone, EGCG alone, and various combinations of these agents. A, HT29 cells were treated with the indicated concentrations of EC alone, EGCG alone, and the indicated combinations of these agents for 48 hours, and cell viability assays were then done using the MTT assay system. *Bars,* SD of triplicate assays. B, combination indices (*CI*) of EGCG plus EC in HT29 cells. The combination indices were determined as previously described (22, 23). The results are summarized in Table 2. C, induction of apoptosis in HT29 cells by EC alone, EGCG alone, and the combination of these agents. The cells were treated with DMSO or 10 μg/mL EGCG in combination with five different doses of EC (0, 1.0, 10, 50, and 100 μg/mL) for 48 hours, and cell extracts were then examined for DNA fragmentation, as described in Materials and Methods. Each asterisk indicates a significant difference ($P < 0.05$) between the control untreated cells (*blank column*) and the catechin-treated cells. *Bars,* SD of triplicate assays.

Table 2. Combined effects of EGCG and EC on HT29 cells

EC concentration (μg/mL)	EGCG concentration (μg/mL)			
	0.1	1	10	20
1	−	−	+	++
10	−	±	+	++
50	±	±	++	+++
100	±	±	++	+++

NOTE: Summary of symbols: −, combination index (CI) 1.1-1.3 (moderate antagonism); ±, CI 0.9-1.1 (additive effect); +, CI 0.8-0.9 (slight synergism); ++, CI 0.6-0.8 (moderate synergism); +++, CI 0.4-0.6 (synergism).

($P < 0.05$) increase in apoptosis (Fig. 6*C*). Indeed, the extent of apoptosis induced by this combination was approximately equivalent to that obtained when the cells were treated with 20 μg/mL of EGCG in the absence of EC (Fig. 3*C*). Thus, a concentration of EC that alone does not induce apoptosis can enhance the apoptotic effect of EGCG. Taken together, the data in Fig. 6 and Table 2 indicate that although EC has weak or no activity when tested alone, when combined with EGCG it can enhance both the growth inhibitory and apoptotic effects of EGCG. The relevance of these combination studies to the relative potencies of EGCG and Poly E, which is a mixture of catechins, is presented in detail in Discussion.

The mechanism by which EC exerts these effects in HT29 cells remains to be determined. It might relate to the cellular uptake of EGCG because Suganuma et al. (19) found that EC enhances cellular incorporation of [^{3}H]EGCG in lung cancer cells. The synergistic effects of the combination of EGCG and EC on growth inhibition and induction of apoptosis of colon cancer cells noted in the above study (Fig. 6; Table 2), and in a previous study of lung cancer cells (19), coupled with our finding that EGCG and Poly E exert similar inhibitory effects on specific signaling pathways (Fig. 4), may help to explain previous studies demonstrating the efficacy of total green tea extracts and Poly E in inhibiting tumor development in rodents (12, 20).

Longer exposure time increases the sensitivity of HT29 cells to (−)-epigallocatechin gallate and Polyphenon E. In clinical studies, the peak plasma concentration of EGCG was in the range of 0.2 to 0.4 μg/mL after administration of 800 mg of EGCG or Poly E (40, 41). This dose is equivalent to drinking about 8 to 16 cups of green tea per day (40, 41). In the above studies, we exposed the colon cancer cells to EGCG or Poly E for a maximum of 48 hours. Because in a clinical setting patients would experience chronic exposure to these compounds, we examined whether we could obtain growth inhibitory effects at lower concentrations by treating the cells with these compounds for 96 hours. We also increased the sensitivity of the growth inhibition assay by directly counting cells in a Coulter counter rather than using the MTT assay, because although 1 μg/mL of EGCG or 1 μg/mL of Poly E caused about 20% inhibition of cell viability in MTT assays (Fig. 1), the same concentration of these compounds caused about 50% inhibition of cell growth when the cells were directly counted in a Coulter counter at 48 hours (Fig. 7*A*). Using these conditions, we found that treatment of HT29 cells with only 1 μg/mL of EGCG or Poly E caused about 65% inhibition of growth at 96 hours (Fig. 7*A*). We found that 1 μg/mL of EGCG or Poly E also caused significant induction of apoptosis (Fig. 7*B*) and a decrease in cellular levels of the p-EGFR and p-HER2 proteins (Fig. 7*C*) when the cells were treated with these agents for 96 hours. These results suggest that in a clinical setting chronic exposure to low serum concentrations of these agents might also inhibit the growth of colon cancer cells (see Discussion).

Discussion

The above results provide the first detailed description of the cellular and molecular effects of EGCG on human colon cancer cells, and a parallel comparison on the effects of Poly E, a mixture of catechins present in green tea. Both materials display similar effects with respect to causing growth inhibition (Figs. 1 and 7*A*; Table 1), arrest of the cell cycle in G_1, induction of caspase-3 and -9, and induction of apoptosis in HT29 cells (Figs. 3 and 7*B*). Both compounds inhibit activation of EGFR and HER2 receptors as well as the downstream effectors ERK and Akt (Fig. 4). They also inhibit the transcriptional activity of the AP-1, c-*fos*, NF-κB, and cyclin D1 promoters in HT29 cells (Fig. 5). These effects are not confined to HT29 cells because in recent studies we have obtained similar results with the SW837 human colon cancer cell line (data not shown). In the present study, we found that EC, a catechin present in Poly E, is a weak inhibitor of growth of HT29 cells. However, when it is combined with EGCG it can cause synergistic inhibition of growth and induction of apoptosis in HT29 cells (Fig. 6; Table 2). The ability of EC to enhance the growth inhibitory and apoptotic effects of EGCG may explain, at least in part, our finding that, although on a weight basis Poly E contains only about 60% EGCG, Poly E had approximately the same IC_{50} value as EGCG in five colon cancer cell lines (Table 1), and was similar to EGCG on a weight basis in its ability to cause an increase of cells in G_1, induce apoptosis, and inhibit activation of EGFR and HER2 and related downstream signaling pathways (Figs. 3 and 4). In the present study, we focused on possible synergistic effects between EGCG and EC on colon cancer cells because of previous evidence that these two compounds exert synergistic effects on inhibiting the growth of PC-9 human lung cancer cells (19). When HT29 cells were treated with Poly E for 48 hours the IC_{50} value was about 20 μg/mL (Fig. 1; Table 1). On a weight basis, EGCG and EC constitute about 60% and 7% of Poly E (20). Therefore, 20 μg/mL of Poly E correspond to about 12 μg/mL of EGCG and 1.4 μg/mL of EC. These concentrations are in the range in which we saw synergistic effects with a combination of EGCG plus EC, with respect to both growth inhibition and induction of apoptosis (Fig. 6; Table 2). Therefore, Poly E may contain optimal relative concentrations of these two compounds. We should, however, emphasize that when we tested low ratios of EC to EGCG, we observed moderate antagonism with respect to growth inhibition (Table 2). Therefore, this aspect must be considered in further studies that combine EGCG with EC or other catechins.

A general question raised by the present and previous studies on EGCG is whether this and related compounds exert growth inhibitory effects on cancer cells by acting on cell surface

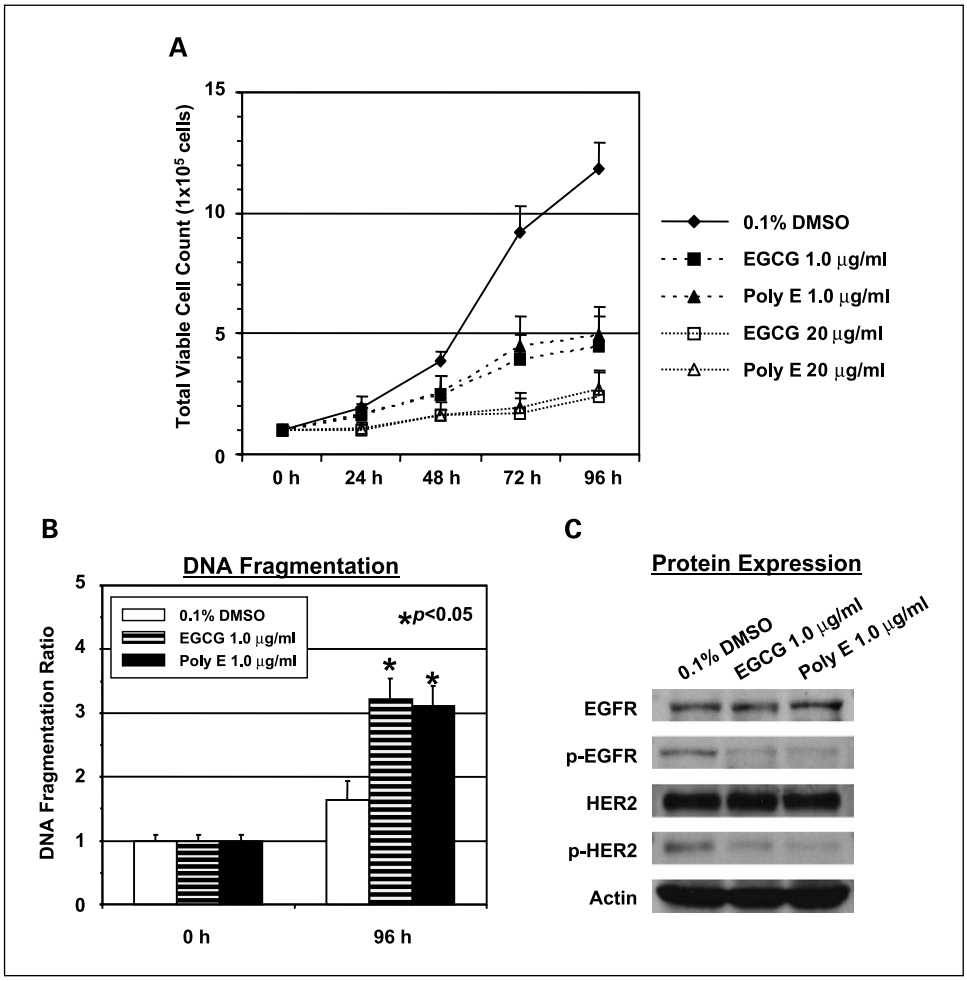

Fig. 7. Effects of low doses of EGCG and Poly E on inhibition of cell growth, induction of apoptosis, and inhibition of EGFR and HER2 activities in HT29 cells. A, cells were treated with 1 μg/mL EGCG, 1 μg/mL Poly E, 20 μg/mL EGCG, 20 μg/mL Poly E, or 0.1% DMSO for 96 hours, and the numbers of cells were then counted at the indicated times using a Coulter Counter. *Points,* means of triplicate assays. B and C, cells were treated with 1 μg/mL EGCG, 1 μg/mL Poly E, or 0.1% DMSO for 96 hours. *B,* cell extracts were examined for DNA fragmentation, as described in Materials and Methods. C, cell extracts were examined by Western blot analysis using the respective antibodies. Each asterisk indicates a significant difference ($P < 0.05$) between the control untreated cells and the EGCG- or Poly E-treated cells. *Bars,* SD of triplicate assays. An antibody to actin served as a loading control. Similar results were obtained in a repeat experiment.

receptor tyrosine kinase receptors and/or through cellular uptake and direct binding to critical intracellular molecules. With respect to targets at the cell surface, previous studies indicate that EGCG can inhibit the binding of EGF, platelet-derived growth factor, and fibroblast growth factor to their respective receptors (15, 42). In addition, EGCG can directly inhibit the tyrosine kinase activity of EGFR (15). These effects may account for the inhibitory effects of EGCG on the autophosphorylation of EGFR in the presence of the respective ligand. In the present study, we showed that EGCG also inhibits activation of both the EGFR and HER2 in HT29 colon cancer cells (Figs. 4 and 7*C*). We previously reported similar effects in human HNSCC and breast cancer cells (16, 17). EGCG inhibition of the activation of HER2 has also been described in murine mammary tumor cells (18). Because in HT29 cells this inhibitory effect on the EGFR and HER2 is a fairly rapid event, occurring within 6 hours (Fig. 4*A*), it could lead to inhibition of several downstream signaling pathways, and thus account for the multiple inhibitory effects of EGCG on signal transduction and gene expression noted in the present and our previous studies (16, 17). Other investigators have also suggested that direct inhibition of the activation of EGFR or HER2 by EGCG could explain the inhibitory effects of this compound on multiple pathways of signal transduction (15, 18, 43). On the other hand, there is evidence that EGCG can directly target intracellular signaling molecules. Thus, EGCG can inhibit AP-1 activity in *Ras*-transformed JB6 cells, which suggests that it can act downstream of EGFR (38). Furthermore, it has been shown that EGCG can inhibit the association between Raf-1 and mitogen-activated protein/ERK kinase 1 (MEK1), cause a decrease in the phosphorylated forms of ERK and MEK1, and directly inhibit the phosphorylation of ELK1 by ERK (44). In addition, Sah et al. (43) found that in immortalized cervical cells EGCG can inhibit EGF-dependent activation of EGFR, but it can also directly inhibit the subcellular kinase activity of ERK and Akt. In a previous study, Chen et al. (45) reported that when HT29 cells were treated with 100 μmol/L (about 46 μg/mL) of EGCG for 4 hours, there was an increase in the phosphorylated forms of the c-jun-NH_2-kinase, ERK, and p38 proteins. The authors suggested that these effects might be due to the oxidative stress caused by this high concentration of EGCG (45). On the other hand, in the present study we found that when HT29 cells were treated with 20 μg/mL of EGCG or Poly E, there was no change in the level of p-ERK for the first 6 hours and thereafter there was a marked decrease in p-ERK (Fig. 4*A*). We assume that the latter effect is more likely to represent the *in vivo* situation because it occurs at a lower concentration of EGCG. A recent study indicates that EGCG can also inhibit DNA methyltransferase activity and, thereby, reactivate the expression of methylation-silenced genes, including the growth inhibitory gene $p16^{INK4a}$ (46). Therefore, additional studies

are required to clarify the direct effects of EGCG on intracellular targets and their relevance to the antitumor effects of this compound. Furthermore, in cell culture and *in vivo*, EGCG can undergo transformation to various derivatives (12, 47), which may also exert both extracellular and intracellular effects. Taken together, the above findings suggest that the antitumor effects of EGCG, and of several other naturally occurring polyphenolic compounds (for review see ref. 48), may be due to binding, probably with relatively low affinity, to multiple cellular targets.

Our finding that at an appropriate dose EGCG and Poly E can inhibit the growth of human colorectal cancer cells without affecting the growth of a normal colon cell line (Fig. 1; Table 1), and our evidence that both EGCG and Poly E exert similar inhibitory effects on critical signaling pathways in colon cancer cells (Fig. 4), should encourage clinical studies with these materials on colon cancer prevention and treatment. Poly E may be preferable to EGCG because it is easier to prepare and because the mixture of catechins in this material may exert synergistic growth inhibitory effects (Fig. 6). An important issue is the question of bioavailability because pharmacokinetic studies in humans indicate that the peak plasma concentration of EGCG is about 0.2 to 0.4 μg/mL (40, 41), whereas in the current cell culture study the IC_{50} concentration of EGCG was about 20 μg/mL (Fig. 1; Table 1). Nevertheless, doses of green tea or doses of EGCG equivalent to those consumed by heavy tea drinkers have been shown to exert antitumor effects in rodents (12, 20). This may reflect the longer duration of exposure and/or tissue accumulation in the intact animal. Indeed, in the present study when we treated HT29 cells for 96 hours rather than 48 hours, 1 μg/mL of EGCG or Poly E was sufficient to inhibit growth, inhibit activation of EGFR and HER2 receptors, and induce apoptosis (Fig. 7). These results were reproducible and not confined to HT29 cells because when SW837 human colon cancer cells were exposed to 1 μg/mL of EGCG for 96 hours, there was about 55% inhibition of cell growth and also inhibition of activation of EGFR and HER2 receptors (data not shown). Further studies are required to determine whether the enhanced effects we obtained with prolonged exposure of colon cancer cells to a low concentration of EGCG or Poly E are due to the continued presence of significant concentration of these compounds or their derivatives (12, 47) in the growth medium or the colon cancer cells, or due to early cellular effects of these compounds that continue to be manifest during subsequent rounds of cell replication.

For the therapy of colon cancer it may be efficacious to combine Poly E with one or more chemotherapy agents because in previous cell culture studies we found that concentrations of EGCG as low as 0.1 to 1 μg/mL exert synergistic growth inhibition of cancer cells when combined with 5-fluorouracil (16), taxol (17), or OSI-461 (data not shown), which is a potent derivative of Apotosyn (sulindac sulfone). It has also been reported that the antitumor activity of the EGFR inhibitor ZD1839 was enhanced when it was combined with the cytotoxic drug oxaliplatin in both HT29 and LoVo human colon cancer cell lines (49). The antitumor activity of ZD1839 was also enhanced when it was combined with the cytotoxic drug paclitaxel in a mouse xenograft model using the GEO human colon cancer cell line (31). It may therefore be of interest to combine Poly E with specific cytotoxic drugs in the treatment of colon cancer.

Acknowledgments

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