Polyphenon E Inhibits the Growth of Human Barrett's and Aerodigestive Adenocarcinoma Cells by Suppressing Cyclin D1 Expression

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Abstract

Purpose: Green tea consumption has been shown to exhibit cancer-preventive activities in preclinical studies. Polyphenon E (Poly E) is a well-defined green tea–derived catechin mixture. This study was designed to determine the effects of Poly E on the growth of human Barrett's and aerodigestive adenocarcinoma cells and the mechanisms involved in growth regulation by this agent.

Experimental Design: Human adenocarcinoma cells and immortalized Barrett's epithelial cells were used as model systems.

Results: Poly E inhibited the proliferation of immortalized Barrett's cells as well as various adenocarcinoma cells, and this was associated with the down-regulation of cyclin D1 protein expression. Inhibition of cyclin D1 led to dephosphorylation of the retinoblastoma protein in a dose-dependent manner; these changes were associated with G_1 cell cycle arrest. Poly E down-regulated cyclin D1 promoter activity and mRNA expression, suggesting transcriptional repression, and this correlated with decreased nuclear β-catenin and β-catenin/TCF4 transcriptional activity. MG132, an inhibitor of 26S proteosome, blocked the Poly E–induced down-regulation of cyclin D1, and Poly E promoted cyclin D1 polyubiquitination, suggesting that Poly E also inhibits cyclin D1 expression by promoting its degradation.

Conclusion: Poly E inhibits growth of transformed aerodigestive epithelial cells by suppressing cyclin D1 expression through both transcriptional and posttranslational mechanisms. These results provide insight into the mechanisms by which Poly E inhibits growth of Barrett's and adenocarcinoma cells, and provides a rationale for using this agent as a potential chemopreventive and therapeutic strategy for esophageal adenocarcinoma and its precursor, Barrett's esophagus.

Esophageal cancer is one of the deadliest cancers in the world and is the seventh leading cause of cancer death among men in the United States (1). In the past several decades, the incidence of esophageal adenocarcinoma has increased at an alarming rate in western countries, including the United States, and has exceeded that of squamous cell carcinoma (2). Esophageal adenocarcinoma is associated with a premalignant condition, Barrett's esophagus (3). Despite the fact that many patients with Barrett's esophagus experience gastroesophageal reflux disease symptoms, many patients who develop esophageal adenocarcinoma present with advanced, metastatic disease at first examination. The overall 5-year survival rate for patients presenting with symptomatic adenocarcinoma is ~13% (4). Because the mortality from this disease remains very high, it is important to identify new agents for the prevention and treatment of esophageal adenocarcinoma and to understand their mechanisms of action.

Some epidemiologic studies have suggested that the consumption of green tea is linked to a decreased incidence of various cancers, including esophageal cancer (5–7). In animal models, green tea or its constituents can inhibit tumor formation induced by multiple carcinogens at various organ sites such as the esophagus, skin, lung, liver, colon, and mammary glands (8–11). Several studies have focused on the effects and mechanism of action of (-)-epigallocatechin gallate (EGCG), a major constituent of green tea, on various cell types (12, 13). Liang et al. (14) reported that EGCG binds to and inhibits epidermal growth factor (EGF)–induced autophosphorylation of EGF receptor and blocks the binding of EGF to its receptor. Shimizu et al. (15, 16) found that EGCG inhibits the activation of EGF receptor, the receptors HER2 and HER3, and their downstream signaling pathways (including extracellular signal–regulated kinase, cyclooxygenase 2, cyclin D1, and

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Translational Relevance

There have been dramatic increases in esophageal adenocarcinoma and its precursor, Barrett's esophagus, during the past decade in western countries. Aerodigestive cancers represent major causes of morbidity and mortality, emphasizing the importance of improved chemoprevention and treatment of these diseases. Green tea consumption has been shown to exhibit cancer-preventive properties in epidemiologic and preclinical studies, but potential mechanisms of action have not been fully elucidated. Polyphenon E is a green tea – derived catechin mixture, which has been shown to have antitumor properties in preclinical studies. We present data indicating that this compound inhibits the growth of human Barrett's epithelial cells and aerodigestive adenocarcinoma cells (including esophageal cancer cells) by suppressing cyclin D1 expression through both transcriptional and posttranslational mechanisms. We believe that these results provide insight into the mechanisms by which polyphenon E may function as a potential chemopreventive and therapeutic agent for esophageal adenocarcinoma and its precursor, Barrett's esophagus.

Bcl-XL) in human colon, breast, and head and neck cancer cell lines.

However, the effects of polyphenon E (Poly E) on tumor cells and its mechanisms of action have been less well studied. Poly E is a well-standardized decaffeinated green tea catechin mixture that contains several polyphenolic compounds, including catechins EGCG, (-)-epigallocatechin, epicatechin-3-gallate, and epicatechin, with EGCG being the most abundant component (11). Because the formulation is highly reproducible and easily prepared, Poly E is an attractive derivative of green tea for clinical chemoprevention trials. It has been used in several clinical studies, including an ongoing clinical phase II study of lung cancer (11) and a phase IB study in patients with Barrett's esophagus (MDA03-1-01). Shimizu et al. (16) recently reported that Poly E has efficacy similar to EGCG in inhibiting human colon cancer cell growth and activation of the EGF receptor and HER2 in human colon cancer cells. It has been suggested that Poly E may be preferable to EGCG because it is easier to prepare and the catechins in this mixture may exert synergistic effects (16). Using the A/J mouse model, Yan et al. (11) reported that Poly E in the diet (2%, w/w) reduced tumor multiplicity by 46% and tumor load by 94% in the benzo[*a*]pyrene-induced lung tumorigenesis model. These data stimulated a phase II clinical chemoprevention trial in individuals with lung cancer (11). However, the potential role of Poly E in the prevention and treatment of human esophageal adenocarcinoma and its mechanisms of action remain to be determined.

The cyclin D1 proto-oncogene controls mechanisms that regulate cell cycle progression by activating cyclin-dependent kinase 4/6 and promoting the phosphorylation of the retinoblastoma protein (Rb) and release of E2F, which drives the cell cycle from the G_1 phase to the S phase (17, 18). Increased cyclin D1 expression or activity as a result of gene amplification or translocation is common in many human cancers, including breast, esophagus, lung, head and neck, colon, and prostate cancers (19). Transgenic mice engineered to overexpress cyclin D1 in mammary glands develop breast cancer, further suggesting a causative role for this protein in tumorigenesis (20). Cyclin D1 overexpression has also been associated with aggressive forms of esophageal cancer (21) and hepatocellular carcinoma (22, 23) and has been associated with both the poor prognosis and chemotherapy resistance of epithelial tumors (24). Cyclin D1 is therefore a promising clinical target for both cancer therapy and prevention.

In this study, we determined the effects of Poly E on human esophageal adenocarcinoma and Barrett's esophagus cells and its mechanisms of action. We found that Poly E inhibits the growth of these cells by down-regulating cyclin D1 expression via both transcriptional and posttranslational mechanisms. These results provide novel insights into the mechanisms of Poly E inhibition of esophageal adenocarcinoma cell growth and provide the rationale for using this agent as a potential chemopreventive and therapeutic strategy for treating esophageal adenocarcinoma and its precursor, Barrett's esophagus.

Materials and Methods

Materials. Poly E produced by Tokyo Food Techno Co. Ltd. was kindly provided by Dr. James A. Crowell (National Cancer Institute, Bethesda, MD). Poly E contains ~53% EGCG, 9% epicatechin, 11% (-)-epigallocatechin, 5% epicatechin-3-gallate, and 5% (-)-gallocatechin gallate. Therefore, 20 μg/mL Poly E contains ~11 μg/mL EGCG, 1.8 μg/mL epicatechin, 2.2 μg/mL (-)-epigallocatechin, and 1 μg/mL (-)-gallocatechin gallate. EGCG was obtained from Sigma Chemical Co. MG132 was obtained from Calbiochem. Lactacystin was obtained from Cayman Chemical. Polyclonal antibodies against cyclin D1, phospho-Rb, and ubiquitin were obtained from Santa Cruz Biotechnology. Polyclonal antibody against β-catenin was purchased from Cell Signaling Technology.

Cell lines and cell cultures. The human adenocarcinoma cell lines SEG-1, BIC-1, SKGT-4, and BE3 were kindly provided by Dr. Uma Raja (The University of Texas M. D. Anderson Cancer Center, Houston, TX) and have been previously described (25, 26). These cell lines have been extensively reported in the literature to be derivatives of human esophageal adenocarcinomas. It has recently been suggested, however, that SEG-1 cells, which have been extensively used as an example of a cell line derived from a Barrett's-associated esophageal adenocarcinoma, may instead represent a lung adenocarcinoma cell line.[3] Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μL/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The h-TERT–immortalized Barrett's esophagus cell lines CP-A and CP-C (kind gift from Dr. Peter Rabinovich, Fred Hutchinson Cancer Center, Seattle, WA, and supplied by Dr. Xiao-Chun Xu, M. D. Anderson Cancer Center, Houston, TX) were grown in MCDB-153 medium supplemented with 5% FCS, 20 ng/mL EGF (Life Technologies), 140 μg/mL bovine pituitary extract, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium (Sigma), as described previously (27). As an untreated solvent control, cells were exposed to DMSO (Sigma) at a final concentration of <0.1%. Cellular cytotoxicity was assessed by cell counts, trypan blue exclusion analysis, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Promega). For trypan blue exclusion analysis, cells were treated with Poly E for 16 h, mixed with 0.4% trypan blue (1:1), and examined under a light microscope for dye exclusion.

[3] Dr. David Beer, personal communication.

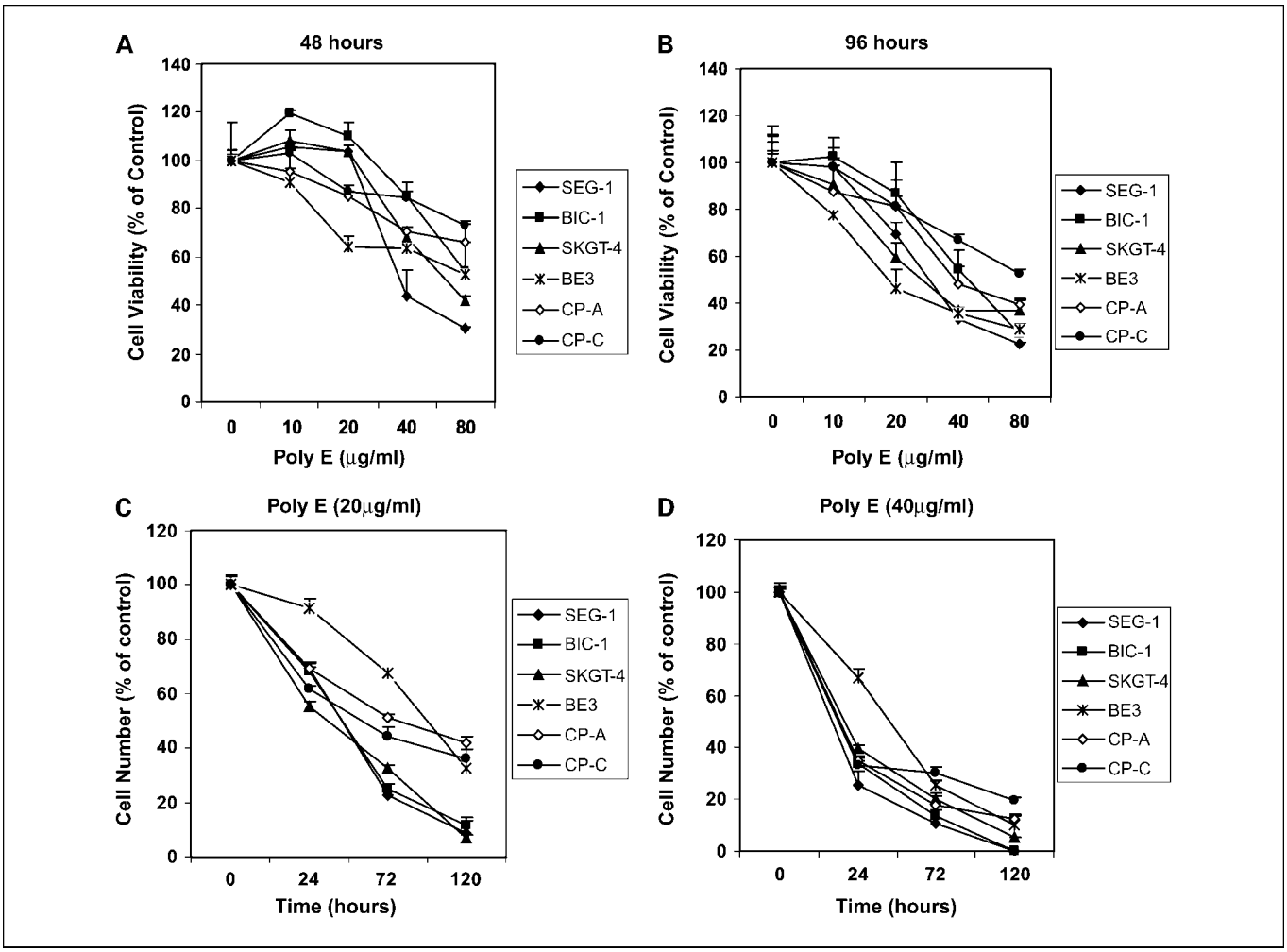

Fig. 1. Inhibition of cell growth by Poly E in human aerodigestive adenocarcinoma cells and immortalized Barrett's cells. Adenocarcinoma SEG-1, BIC-1, SKGT-4, and BE3 cells and immortalized Barrett's CP-A and CP-C cells were seeded in 96-well plates and treated with up to 80 μg/mL Poly E for 48 h (*A*) or 96 h (*B*). A nonradioactive MTS cell proliferation assay was done to determine the rate of proliferation. Esophageal adenocarcinoma SEG-1, BIC-1, SKGT-4, and BE3 cells and immortalized Barrett's CP-A and CP-C cells were seeded in six-well plates and treated with 20 μg/mL Poly E (*C*) or 40 μg/mL Poly E (*D*) for 0 (control), 24, 72, or 120 h. Cells were counted using a Coulter counter to determined cell survival. Points, mean of triplicate experiments; bars, SD.

Cell proliferation assays. The affect of Poly E on cell growth was assessed by the 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay and by direct cell counting. Cell viability assays were done using the CellTiter 96 aqueous nonradioactive cell proliferation assay (MTS) according to the instructions of the manufacturer (Promega). SEG-1, BIC-1, SKGT-4, BE3, and CP-A, CP-C cells were seeded onto 96-well plates (3×10^3 per well). Twenty-four hours later, the cells were treated with up to 80 μg/mL Poly E for 48 or 96 h in DMEM. The medium and reagents were not changed during this time period. As an untreated solvent control, cells were treated with DMSO (Sigma Chemical) at a final concentration of <0.1%. All assays were done in triplicate. Cell numbers were also directly assessed by cell counting. Esophageal adenocarcinoma SEG-1, BIC-1, SKGT-4, and BE3 cells and Barrett's esophagus CP-A and CP-C cells were seeded onto six-well plates in DMEM and, 24 h later, treated with 20 or 40 μg/mL Poly E for 24, 72, or 120 h. The medium and reagents were changed once at 72 h. As a control, cells were treated with 0.1% DMSO, replicate plates of cells were washed with PBS, and the attached cells were collected by trypsinization. The number of cells was assayed in triplicate using a Coulter counter (Beckman Coulter).

Flow cytometric analysis. SEG-1, BIC-1, and SKGT-4 cells were seeded onto six-well plates (4×10^5 per well) in DMEM and cultured for 24 h to allow cell attachment. The cells were then treated with 0.1% DMSO or with 20 or 40 μg/mL Poly E for 24, 48, or 72 h. The cells were then harvested, fixed with methanol, washed, treated with RNase A, and stained for DNA with propidium iodide (Sigma) and then were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry using a FACSCalibur instrument (Becton Dickinson); the data were analyzed using the CELL Quest computer program (Becton Dickinson). To determine whether the treated cells underwent apoptosis, cells treated with up to 100 μg/mL Poly E for 36 h were washed in PBS, resuspended in 100 μL of binding buffer containing FITC-conjugated Annexin V, and analyzed by flow cytometry to determine the apoptosis index.

Protein extraction and Western blot analysis. Cells were lysed in buffer containing 30 mmol/L Tris-HCl (pH 6.8), 150 mmol/L NaCl, 2 mmol/L EDTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 2 mmol/L orthovanadate, 1% Triton X-100, 1% NP40, 0.2 mmol/L phenylmethylsulfonyl fluoride, and one mini-tablet of protease inhibitor cocktail (Roche Diagnostics). The protein concentration of supernatant was determined using the bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein were subjected to electrophoresis on 10% Tris-glycine gels. After gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane, which was stained with 0.5% Ponceau S with 1% acetic acid to confirm equal

loading and transfer efficiency. Western blot analysis was done by incubating the membranes at room temperature for 1 h in a blocking solution containing 5% nonfat dry milk and 0.1% Tween 20 in TBS [10 mmol/L Tris-HCl with 150 mmol/L NaCl (pH 7.6)], probing with specific primary antibodies, washing with TBS containing 0.1% Tween 20, and finally probing with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized using a chemiluminescence detection technique.

Immunocytochemistry. For immunocytochemical staining, cells were harvested and washed twice with PBS, and the cell concentration was adjusted to 5×10^5/mL. A cytospin cytologic centrifuge (Shandon Cytospin 4, Thermo electron Corp.) was used to make cell preparations of 100- to 200-μL cell suspensions, which were then air-dried and fixed with 4% paraformaldehyde for 30 min at room temperature and washed with 0.1% Triton X-100 in PBS. Cyclin D1 and phospho-Rb were detected with 1 μg/mL anti–cyclin D1 and anti–phospho-Rb antibody, respectively. As a control, primary antibody was substituted with normal rabbit immunoglobulin.

Coimmunoprecipitation. Cells were lysed in immunoprecipitation buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Igepal, 1 μL of protease inhibitor cocktail, and 1 μmol/L phenylmethylsulfonyl fluoride. Lysates then underwent three cycles of freezing and thawing to lyse the nuclei. Total cell lysates (800 μg of protein) were incubated with 2 μg of anti–cyclin D1 antibody or normal rabbit immunoglobulin. Proteins associated with cyclin D1 were precipitated using protein A/G-agarose beads (Santa Cruz Biotechnology). Immunocomplexes were analyzed by SDS-PAGE, and associated proteins were detected by immunoblotting using the enhanced chemiluminescence protein detection system (Amersham).

Plasmids, transient transfection, and luciferase reporter assays. Cyclin D1 promoter luciferase reporter (19) and SuperTop TCF4 luciferase reporter were kindly provided by Dr. X-H. Feng (Baylor College of Medicine, Houston, TX) and Dr. Chunming Liu (The University of Texas Medical Branch, Galveston, TX). Plasmids were prepared using the Genopure plasmid midi kit from Roche Diagnostics. β-Galactosidase expression vector pCH110 (Amersham-Pharmacia) was used to normalize transfection efficiency.

For transient transfection, cells were seeded at a concentration of 8×10^5 per well in six-well plates. After overnight culture, the cells in each well were transfected with DNA (1 μg of cyclin D1 promoter or SuperTop TCF4 luciferase reporter plasmid and 0.2 μg of pCH110) mixed with 3 μL of FuGENE 6 (Roche Diagnostics) according to the manufacturer's protocol. Cells were cultured for an additional 48 h and harvested for measurement of luciferase activity (in relative light units normalized to β-galactosidase) with a TD-20/20 luminometer (Turner Designs) using the Promega luciferase assay system. The

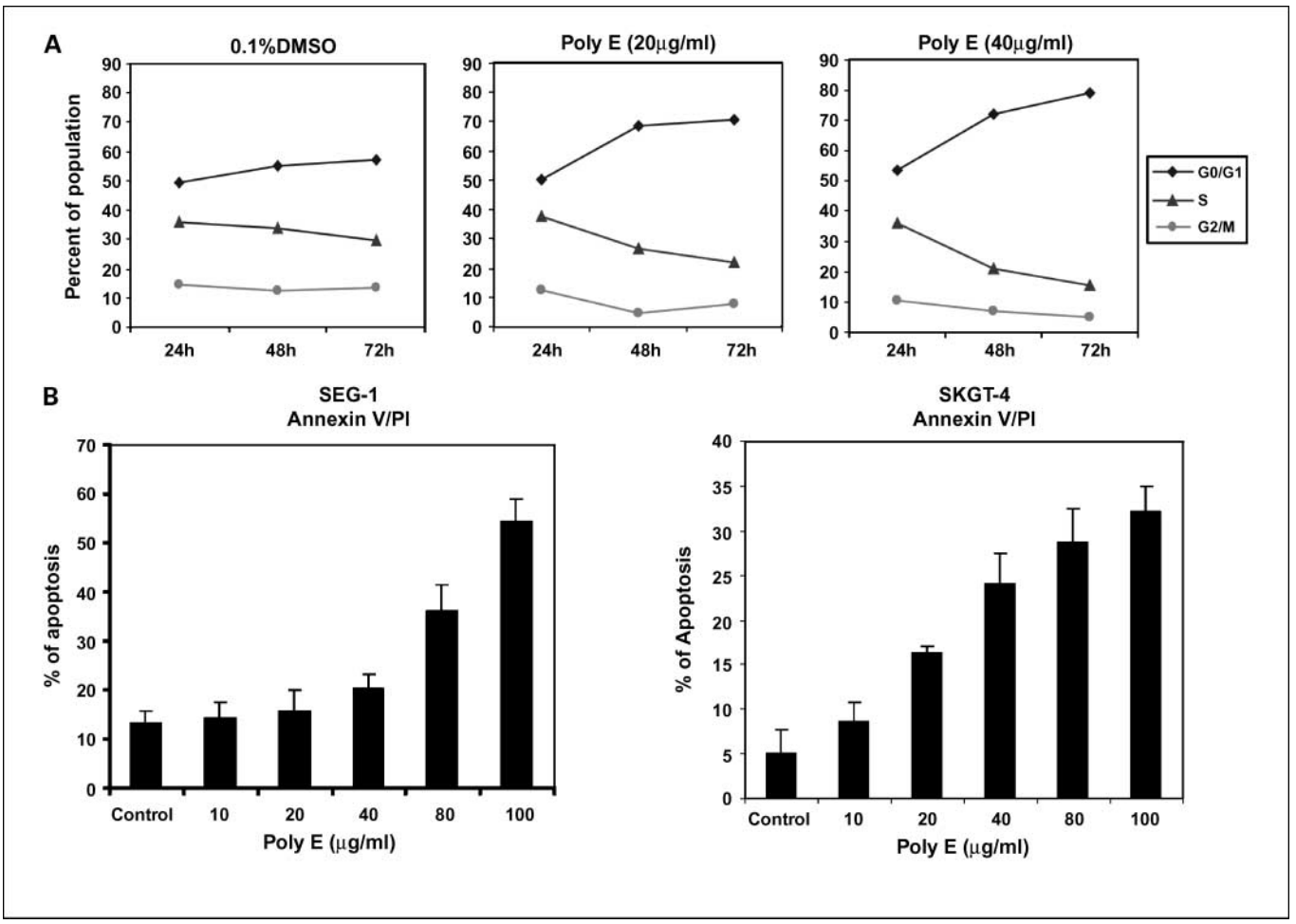

Fig. 2. Poly E arrests adenocarcinoma cells in the G_0-G_1 phase of the cell cycle and induces apoptosis. *A,* SEG-1 cells were seeded in six-well plates and treated with 0.1% DMSO or with 20 or 40 μg/mL Poly E for up to 72 h. The cells were fixed and stained for DNA with propidium iodide and then analyzed for DNA histograms and cell cycle phase distribution by flow cytometry using a FACSCalibur instrument. *B,* SEG-1 and SKGT-4 cells were treated with up to 100 μg/mL Poly E, resuspended in 100 μL of binding buffer containing FITC-conjugated Annexin V, and analyzed by flow cytometry to determine the apoptosis index.

values represent the mean and SD of at least three independent experiments.

Real-time PCR. To determine the fold change in RNA targets, real-time reverse transcription-PCR was done on an ABI Prism 7900 (Applied Biosystems) using the commercially available gene expression assay for cyclin D1, CCND1 (Hs00277039_m1), and the cyclophilin A Vic–labeled predeveloped assay reagent (4326316E; Applied Biosystems) without multiplexing. A 25-μL final reaction volume containing 1× TaqMan Universal PCR master mix (Applied Biosystems), 1× Multiscribe with RNase inhibitors, and 1× gene expression assay was used to amplify ~50 ng of total RNA with the following cycling conditions: 30 min at 48°C, 10 min at 95°C, and 50 cycles at 95°C for 15 s and 60°C for 1 min. The 7900 Sequence Detection System 2.2 software automatically determined the fold change for CCND1 in each sample by using the $\delta\delta C_t$ method with 95% confidence.

Statistical analysis. Statistical significance for the reporter assays was determined using the Student *t* test. Results were considered statistically significant if $P < 0.05$.

Results

Poly E inhibits the growth of human Barrett's esophagus and aerodigestive adenocarcinoma cells. EGCG, the major catechin in green tea, inhibits the growth of human head and neck, breast, and colon cancer cells (14, 15, 28). However, evidence for the effects of Poly E on tumor cell growth, especially Barrett's esophagus and esophageal adenocarcinoma cell growth, is lacking. We determined the effects of Poly E on the growth of two Barrett's esophagus cells and several aerodigestive adenocarcinoma cell lines by using a nonradioactive MTS cell proliferation assay. We observed a dose- and time-dependent decrease in the rate of proliferation in esophageal adenocarcinoma SEG-1, BIC-1, SKGT-4, and BE3 cells and immortalized Barrett's CP-A and CP-C cells (Fig. 1A and B). To confirm the effect of Poly E on growth of Barrett's esophagus

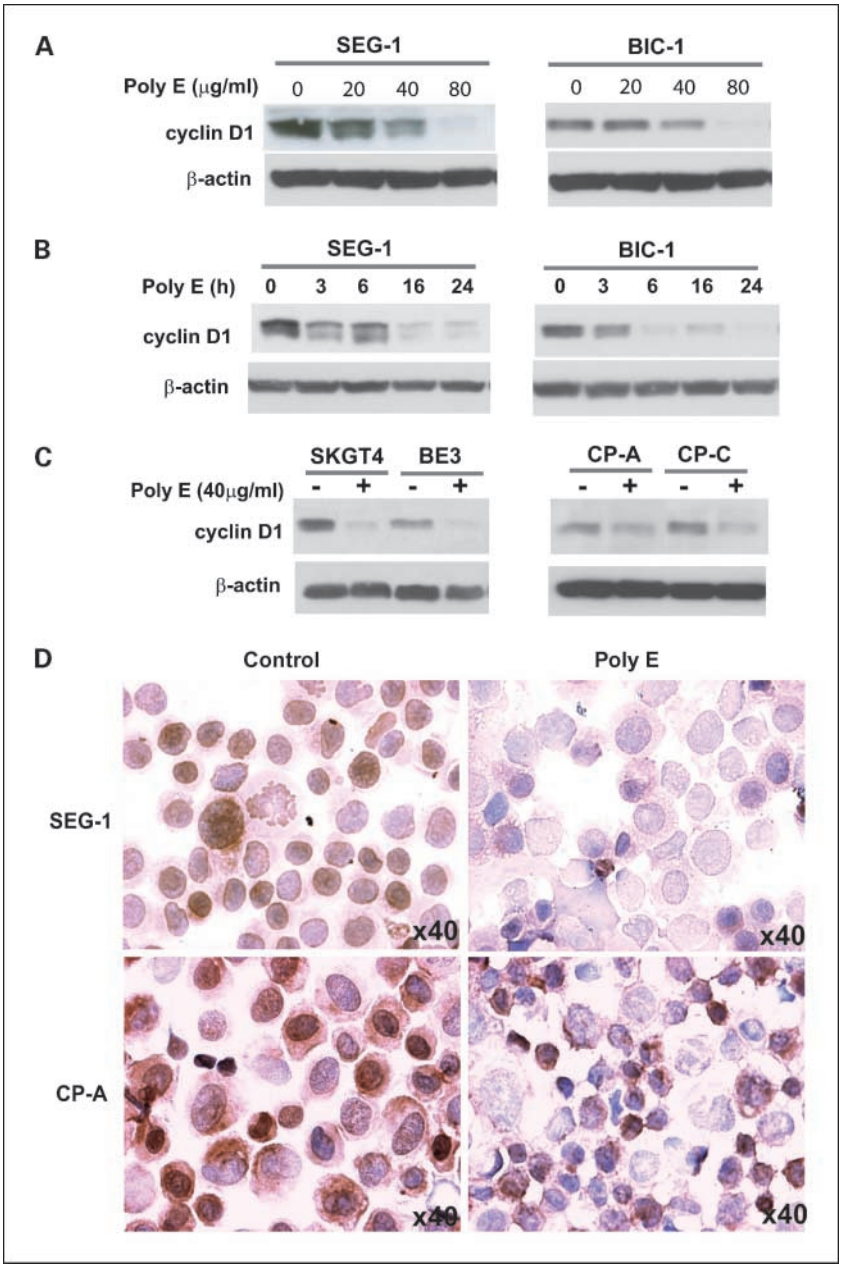

Fig. 3. Poly E inhibits cyclin D1 expression in adenocarcinoma and Barrett's esophagus cell lines. *A,* SEG-1 and BIC-1 adenocarcinoma cells were treated with up to 80 μg/mL Poly E for 24 h and immunoblots were done with anti – cyclin D1 and β-actin antibodies. *B,* SEG-1 and BIC-1 cells were treated with 40 μg/mL Poly E for up to 24 h and immunoblots were done with the same antibodies. *C,* SKGT-4 and BE3 esophageal adenocarcinoma cells and CP-A and CP-C cells were treated with 40 μg/mL Poly E for 16 h and immunoblots were done with anti – cyclin D1 and β-actin antibodies. *D,* SEG-1 and CP-A cells were treated with no Poly E (control) or with 40 μg/mL Poly E for 24 h and prepared in cytospin slides. Immunohistochemical staining was done for cyclin D1 as described in Materials and Methods.

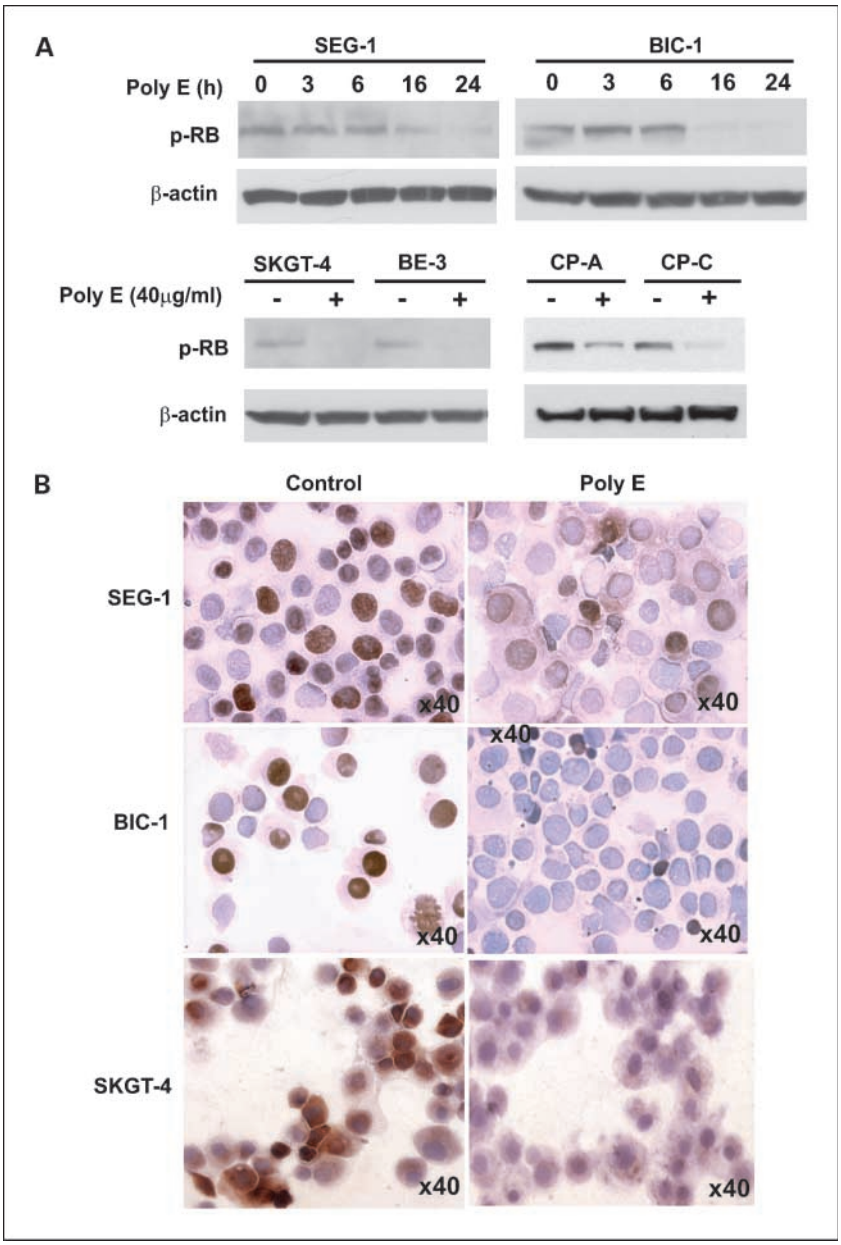

Fig. 4. Poly E dephosphoylates Rb in aerodigestive adenocarcinoma cells. *A,* SEG-1 and BIC-1 cells (*top*) were treated with 40 μg/mL Poly E for up to 24 h, and immunoblots were done to analyze Rb phophorylation status with antibody against phospho-Rb. β-Actin was used as an internal control. SKGT-4 and BE3 cells (*bottom left*) and CP-A and CP-C cells (*bottom right*) were treated with 40 μg/mL Poly E for 24 h and immunoblots were done to analyze Rb phophorylation status with antibody against phospho-Rb. β-Actin was used as an internal control. *B,* SEG-1, BIC-1, and SKGT-4 cells were treated with no Poly E (control) or with 40 μg/mL Poly E for 24 h and prepared in cytospin slides. Immunohistochemical staining was done with antibody against phospho-Rb as described in Materials and Methods.

and adenocarcinoma cells, we performed cell counts over time in the presence of Poly E for SEG-1, BIC-1, SKGT-4, and BE3 cells and CP-A and CP-C cells as described in Materials and Methods. For all these cell lines, time-dependent inhibition was observed at the doses of 20 and 40 μg/mL Poly E tested (Fig. 1C and D).

Poly E arrests adenocarcinoma cells in the G_0-G_1 phase of the cell cycle and induces apoptosis. Poly E can induce the arrest of the cell cycle at the G_1 phase and induce apoptosis in human HT29 colon cancer cells (16). To determine whether the growth inhibition observed in aerodigestive adenocarcinoma cell lines is associated with specific changes in cell cycle distribution, we analyzed the cell cycle using DNA flow cytometry. When SEG-1 cells were treated with 20 or 40 μg/mL Poly E for up to 72 hours, the percentage of cells in the G_0-G_1 phase increased in a dose- and time-dependent manner and was associated with a concomitant decrease of cells in the S phase (Fig. 2A). Similar results for cell cycle arrest were observed for BIC-1 and SKGT-4 cells (data not shown). Furthermore, treatment with Poly E for 36 hours induced apoptosis in SEG-1 and SKGT-4 cells, as indicated by a dose-dependent increase in the apoptosis index using Annexin V and propidium iodide (Fig. 2B).

Poly E inhibits cyclin D1 expression in adenocarcinoma and Barrett's esophagus cell lines. The transition of cells from the G_1 phase to the S phase is in part regulated by cyclin D1. Whether Poly E exerts its effects on the proliferation and cell cycle of esophageal adenocarcinoma cells by regulating cyclin D1 was examined. SEG-1 and BIC-1 cells were treated with up to 80 μg/mL Poly E for 24 hours and then examined for expression of cyclin D1 by Western blot analysis. Poly E down-regulated cyclin D1 in a dose-and time-dependent manner in both cell lines (Fig. 3A and B).These findings were not cell line specific because Poly E also reduced cyclin D1 expression in other transformed esophageal cell lines, including CP-A and CP-C derived from Barrett's epithelium (Fig. 3C).

Immunohistochemical staining of cyclin D1 in cytospin slides confirmed that Poly E dramatically decreased nuclear cyclin D1 staining in SEG-1 and CP-A cells without changes in morphology (Fig. 3D).

Poly E dephosphorylates Rb in Barrett's esophagus and adenocarcinoma cells. Rb tumor suppressor is a direct target of the cyclin D1/cyclin-dependent kinase 4 complex, and the status of Rb phosphorylation determines whether a cell will transition from the G_1 phase to the S phase. We therefore determined whether Poly E affects Rb phosphorylation in Barrett's esophagus and esophageal adenocarcinoma cell lines. Poly E dephosphorylated Rb in a time-dependent manner in SEG-1, BIC-1, SKGT-4, and BE3 esophageal adenocarcinoma cells and in CP-A and CP-C immortalized Barrett's epithelial cells (Fig. 4A).

Immunocytochemical staining with anti–phospho-Rb antibody confirmed that the level of phospho-Rb expression dramatically decreased when the cells were treated with Poly

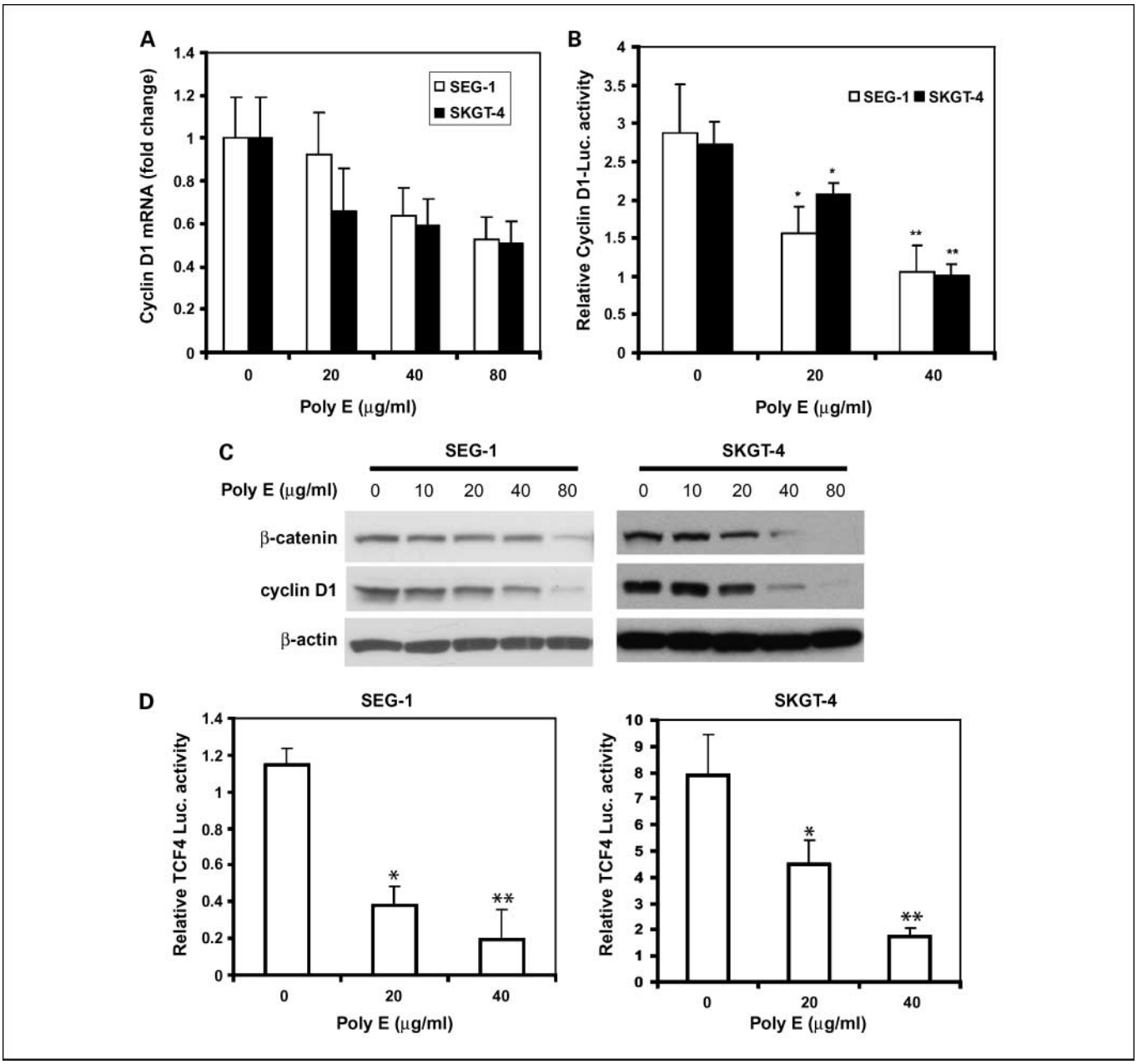

Fig. 5. Poly E down-regulates cyclin D1 transcription in aerodigestive adenocarcinoma cells and decreases β-catenin/TCF4 activity. *A,* SEG-1 and SKGT-4 cells were treated with up to 80 μg/mL Poly E for 24 h, and total RNA was isolated and subjected to real-time reverse transcription-PCR on an ABI Prism 7900 using cyclin D1 primers and probe (CCND1; Hs00277039_mL), with cyclophilin A as internal control. *B,* SEG-1 and SKGT-4 cells were cotransfected with 1 μg of human cyclin D1 promoter and 0.2 μg of pCH110 and then challenged with 20 or 40 μg/mL Poly E or with vehicle (0.1% DMSO). Luciferase reporter activity was measured after 24 h. Columns, mean of at least three independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$, versus no treatment with Poly E. *C,* SEG-1 and SKGT-4 cells were treated with up to 80 μg/mL Poly E for 24 h and immunoblots were done to analyze β-catenin, cyclin D1, and β-actin expression levels. *D,* SEG-1 and SKGT-4 cells were cotransfected with 1 μg of SuperTopTCF4 luciferase reporter and 0.2 μg of pCH110 and then challenged with 20 or 40 μg/mL Poly E or vehicle (0.1% DMSO). Luciferase reporter activity was measured after 24 h. Columns, mean of at least three independent experiments; bars, SD. *, $P < 0.01$; **, $P < 0.001$, versus treatment with vehicle.

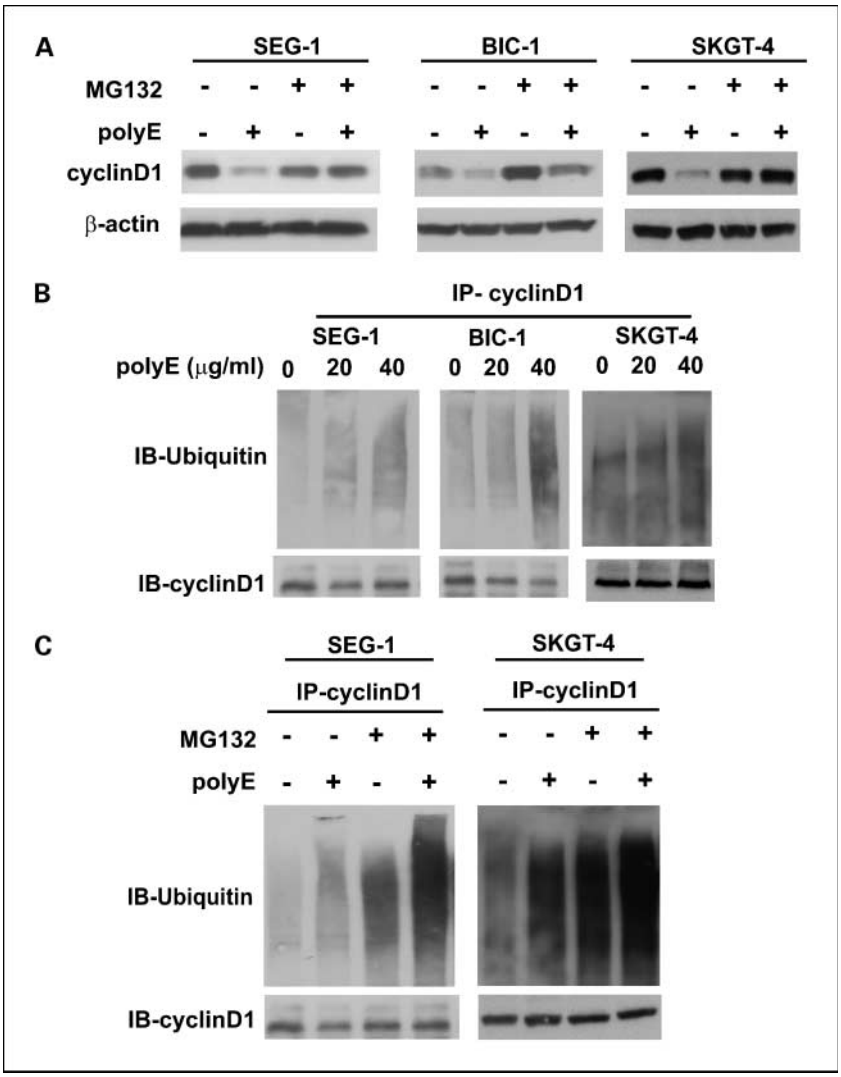

Fig. 6. Poly E enhances cyclin D1 ubiquitination and degradation in adenocarcinoma cells. *A,* SEG-1, BIC-1 and SKGT-4 cells were incubated with 10 μmol/L MG132 for 2 h and then exposed to 40 μg/mL Poly E for 16 h. Immunoblots were done to analyze cyclin D1 and β-actin expression levels. *B,* SEG-1, BIC-1, and SKGT-4 cells were treated with up to 40 μg/mL Poly E for 24 h; 500 μg of whole-cell lysates were immunoprecipitated (*IP*) with anti – cyclin D1 antibody; and 30 μL of protein A/G-agarose beads were added to the immunoprecipitation mixture. Immunoblots (*IB*) were done using antibodies against polyubiquitin and cyclin D1. *C,* SEG-1 and SKGT-4 cells were incubated with 10 μmol/L MG132 for 2 h and then exposed to 40 μg/mL Poly E for 24 h. Whole-cell lysates were immunoprecipitated with anti – cyclin D1 antibody as described in *B*.

E (Fig. 4B). These findings suggest that poly E inhibits the growth of Barrett's esophagus and aerodigestive adenocarcinoma cells, at least in part, by down-regulating cyclin D1 and that it is accompanied by dephosphorylation of Rb.

Poly E down-regulates cyclin D1 transcription, which correlates with decreased β-catenin/TCF4 activity. We next sought to define the mechanisms by which Poly E down-regulates cyclin-D1 expression. We first explored the possibility that Poly E decreases cyclin D1 expression by altering transcription. SEG-1 and SKGT-4 cells were treated with Poly E, and total RNA was isolated and probed and primed for cyclin D1 mRNA using quantitative real-time PCR. A dose-dependent decrease of cyclin D1 mRNA was observed when esophageal adenocarcinoma cells were treated for 24 hours with Poly E (Fig. 5A).

We then explored whether Poly E affects cyclin D1 expression by affecting its promoter activity. SEG-1 and SKGT-4 cells were transfected with cyclin D1 promoter luciferase reporter, treated with 20 or 40 μg/mL Poly E, and assayed for luciferase reporter activity. Poly E decreased cyclin D1 reporter activity in a dose-dependent manner in SEG-1 and SKGT-4 cells (Fig. 5B). These results suggest that Poly E down-regulates cyclin D1 expression, at least in part, by suppressing its transcription.

To further determine which nuclear factors mediate the down-regulation of cyclin D1 by Poly E, we examined nuclear β-catenin and TCF4 activity because cyclin D1 is a downstream target of the Wnt/β-catenin pathway (29), and it has been reported that the level of nuclear β-catenin in small intestinal tumors from EGCG-treated $Apc^{min/-}$ mice is significantly lower than that of untreated controls (30). Treatment of SEG-1 and SKGT-4 cells with Poly E for 24 hours decreased nuclear level of β-catenin in a dose-dependent manner, which corresponded with the decreased cyclin D1 expression (Fig. 5C). The luciferase reporter SuperTop Flash contains eight copies of TCF-binding sites, which have a minimal thymidine kinase promoter and a luciferase open reading frame, and has been recently used to characterize the transcriptional activity of β-catenin/TCF4. SEG-1 and SKGT-4 cells were transfected with the SuperTop Flash construct, treated with 20 or 40 μg/mL Poly E, and assayed for luciferase reporter activity. Poly E inhibited TCF4 activity in a dose-dependent manner (Fig. 5D). These results suggest that Poly E inhibits nuclear β-catenin and β-catenin/TCF4 transcriptional activity, which may mediate down-regulation of cyclin D1.

Poly E also down-regulates cyclin D1 by promoting its degradation. Because cyclin D1 undergoes ubiquitin-dependent

proteosomal degradation (31), it is possible that poly E could down-regulate cyclin D1 by enhancing its degradation. We first examined the ability of MG132, a proteosome inhibitor, to block Poly E–induced degradation of cyclin D1 in SEG-1, BIC-1, and SKGT-4 cells. Cells were pretreated with 10 μmol/L MG132 for 2 hours and then treated with 40 μg/mL Poly E for 16 hours. Whole-cell extracts were prepared and analyzed for cyclin D1 protein. MG132 prevented the decrease in cyclin D1 associated with Poly E treatment in SEG-1, BIC-1, and SKGT-4 cells (Fig. 6A). Similar results were obtained with the selective proteosome inhibitor lactacystin (Supplementary Fig. S1). To explore the possibility that Poly E enhances cyclin D1 polyubiquitination and facilitates its degradation by proteosomes, we performed immunoprecipitation with anti–cyclin D1 antibody and immunoblotting with anti-ubiquitin antibody. Cyclin D1 polyubiquitination was enhanced in a dose-dependent manner when cells were exposed to Poly E (Fig. 6B), and MG132 further enhanced this polyubiquitination (Fig. 6C). These results suggest that Poly E enhances cyclin D1 proteolysis by facilitating cyclin D1 polyubiquitination and its subsequent degradation.

Discussion

Green tea has been shown to be chemopreventive in several animal models of tumorigenesis (8–11). We have shown that Poly E, a well-standardized green tea catechin mixture, potently inhibits the proliferation of aerodigestive adenocarcinoma cells and immortalized Barrett's epithelial cells and that this inhibition correlates with the down-regulation of cyclin D1 protein, dephosphorylation of Rb, and changes associated with cell cycle arrest at the G_1 phase. Our data suggest that Poly E down-regulates cyclin D1 by inhibiting its transcription, which correlates with Poly E–induced decreases in nuclear β-catenin levels and decreased β-catenin/TCF4 transcriptional activity. In addition, Poly E promotes the ubiquitinition of cyclin D1 and its subsequent degradation.

These findings are in agreement with a previous report that Poly E suppresses the proliferation of human colon cancer cells (16). The mechanisms behind the antiproliferative effects of Poly E, however, have not been previously demonstrated. Cyclin D1 has been shown to be overexpressed in many cancers, including esophagus, breast, head and neck, and prostate cancers (32–35). This protein is required for the activity of cyclin-dependent kinase 4/6, which phosphorylates Rb protein, thus releasing E2F to mediate the transition from the G_1 phase to the S phase, which in turn leads to DNA synthesis and cell cycle progression (17). Our data indicate that Poly E inhibits the phosphorylation of Rb and induces cellular arrest in G_1, mediated through the down-regulation of cyclin D1.

Our results suggest that there are at least two mechanisms by which Poly E down-regulates cyclin D1. The first mechanism involves transcriptional repression: Cyclin D1 mRNA and promoter activity were down-regulated by Poly E, suggesting transcriptional repression. Nuclear β-catenin level was suppressed by Poly E, which correlated with cyclin D1 down-regulation. Suppression of β-catenin/TCF4 promoter activity by Poly E was also observed, suggesting that the Wnt pathway mediates transcriptional down-regulation of cyclin D1 by Poly E. These data are consistent with those of Ju et al. (30), who reported that EGCG decreases levels of nuclear β-catenin and its target gene c-*myc* in HT29 human colon cancer cells and small intestinal tumor tissues. Recently, Kim et al. (36) reported that EGCG suppresses TCF4 activity in invasive breast cancer cells by up-regulating the HBP1 transcriptional repressor of Wnt signaling. We were unable to show a similar effect on HBP1 protein levels by Poly E in the cell lines used in our study (data not shown).

Cyclin D1 is degraded through the ubiquitin-dependent proteosomal pathway (31). Our results indicate that Poly E–induced down-regulation of cyclin D1 also occurs by this mechanism because MG132, a proteosomal inhibitor (37), blocked Poly E–induced down-regulation of cyclin D1 expression. These results are similar to those previously reported with curcumin, another chemopreventive agent, which down-regulates cyclin D1 by promoting proteolysis (19). Poly E also promoted cyclin D1 polyubiquitination in a dose-dependent manner, further suggesting that Poly E down-regulates cyclin D1 by promoting its polyubiquitination and, subsequently, its proteosomal degradation.

In conclusion, our results showed that Poly E, a green tea derivative, potently inhibits the growth of human aerodigestive adenocarcinoma cells as well as Barrett's cells by down-regulating cyclin D1 expression via both transcriptional and posttranslational mechanisms. These findings provide insight into the mechanisms by which natural products such as green tea–derived catechins may alter epithelial proliferation in the esophagus, and provide a rationale for these agents as potential chemopreventive or therapeutic modalities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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