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Green tea polyphenol, (−)-epigallocatechin-3-gallate, induces toxicity in human skin cancer cells by targeting β-catenin signaling

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ABSTRACT

The green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCG), has been shown to have anti-carcinogenic effects in several skin tumor models, and efforts are continued to investigate the molecular targets responsible for its cytotoxic effects to cancer cells. Our recent observation that β-catenin is upregulated in skin tumors suggested the possibility that the anti-skin carcinogenic effects of EGCG are mediated, at least in part, through its effects on β-catenin signaling. We have found that treatment of the A431 and SCC13 human skin cancer cell lines with EGCG resulted in reduced cell viability and increased cell death and that these cytotoxic effects were associated with inactivation of β-catenin signaling. Evidence of EGCG-induced inactivation of β-catenin included: (*i*) reduced accumulation of nuclear β-catenin; (*ii*) enhanced levels of casein kinase1α, reduced phosphorylation of glycogen synthase kinase-3β, and increased phosphorylation of β-catenin on critical serine$^{45,33/37}$ residues; and (*iii*) reduced levels of matrix metalloproteinase (MMP)-2 and MMP-9, which are down-stream targets of β-catenin. Treatment of cells with prostaglandin E2 (PGE_2) enhanced the accumulation of β-catenin and enhanced β-catenin signaling. Treatment with either EGCG or an EP2 antagonist (AH6809) reduced the PGE_2-enhanced levels of cAMP, an upstream regulator of β-catenin. Inactivation of β-catenin by EGCG resulted in suppression of cell survival signaling proteins. siRNA knockdown of β-catenin in A431 and SCC13 cells reduced cell viability. Collectively, these data suggest that induction of cytotoxicity in skin cancer cells by EGCG is mediated by targeting of β-catenin signaling and that the β-catenin signaling is upregulated by inflammatory mediators.

Introduction

The risk of cancer continues to grow with the constant rise in life expectancy and detrimental changes in dietary habits, life style and environmental conditions. Skin cancers represent the most common malignant neoplasms in humans, especially in Caucasians. In the United States alone, more than 2 million people are diagnosed with melanoma and non-melanoma skin cancers annually (Am Cancer Soc, 2010) with the incidence of skin cancer being equivalent to the combined incidence of malignancies of all other organs (Housman et al., 2003). Cutaneous malignancies represent a major public health problem and a burden on healthcare expenditures. The use of sunscreens does not adequately protect the skin from the risk of cutaneous malignancies caused by constant exposure to solar ultraviolet radiation. Thus, there is an urgent need for the development of effective therapeutic agents and more effective preventive strategies.

Certain phytochemicals are emerging as promising anti-cancer agents due to their therapeutic activity against many cancers. One such phytochemical is (−)-epigallocatechin-3-gallate (EGCG), an active component of green tea polyphenols. EGCG has been shown to have preventive effects against carcinogenesis in skin cells (Katiyar et al., 2007; Mantena et al., 2005; Meeran et al., 2006). It possesses a wide range of biochemical and pharmacological activities, including antioxidant (Katiyar and Elmets, 2001; Katiyar et al., 2001), anti-inflammatory (Katiyar et al., 1999) and anti-angiogenic (Katiyar et al., 2007) effects that have been demonstrated both *in vitro* and *in vivo* using animal models. It also has been reported that EGCG inhibits UV radiation-induced skin tumorigenesis as well as chemical carcinogen-induced and tumor promoter-promoted skin cancer in animal models (Katiyar et al., 1992, 2007; Mantena et al., 2005; Meeran et al., 2006). The recent finding that β-catenin is overexpressed in UVB-exposed keratinocytes and UVB-irradiated mouse skin (Smith et al., 2012) has focused attention on β-catenin and its associated pathways as candidate therapeutic targets for the treatment or prevention of skin cancers. β-Catenin is an important component of the Wnt pathway. Wnt/β-catenin

Abbreviations: COX-2, cyclooxygenase-2; cAMP, 3′–5′-cyclic adenosine monophosphate; CDK, cyclin-dependent kinase; EGCG, (−)-epigallocatechin-3 gallate; MMP, matrix metalloproteinase; PGs, prostaglandins.

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signaling proteins regulate various target genes that are involved in cellular proliferation and migration. Activation and alterations in Wnt/β-catenin proteins and mutations in β-catenin have been associated with aggressive tumor progression/growth and cancer cell metastasis (Gavert and Ben-Ze'ev, 2007; Klaus and Birchmeier, 2008; Rimm et al., 1999; Vaid et al., 2011a, 2011b). In the canonical model of Wnt signaling, β-catenin activity is regulated by its phosphorylation at certain key residues by casein kinase 1α (CK1α) and glycogen synthase kinase-3β (GSK-3β). These phosphorylation events lead to its ubiquitination and subsequent degradation (Gavert and Ben-Ze'ev, 2007; Klaus and Birchmeier, 2008). Loss of appropriate regulation of β-catenin results in its accumulation in the nucleus and subsequent stimulation of downstream targets that promote cell proliferation and tumor growth (Li et al., 2005; Lowy et al., 2006). We therefore undertook an examination of the effects of EGCG on β-catenin to determine whether β-catenin is a molecular target of EGCG and a possible molecular target for skin cancer chemoprevention. We have assessed the chemotherapeutic effects of EGCG on β-catenin and associated signaling molecules using the A431 and SCC13 human skin cancer cell lines as *in vitro* models. In this study, we show that EGCG inhibits cellular proliferation and induces cell death in A431 and SCC13 human skin cancer cells by targeting β-catenin and its signaling molecules.

Materials and methods

Antibodies and reagents. Antibodies specific for cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), PGE_2 receptor EP2, and associated secondary antibodies and human-specific COX-2 and β-catenin siRNA transfection reagent kits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The EP2 antagonist (AH6809) and antibodies specific for phosphatidylinositol-3 kinase (PI3K), β-catenin, cyclin D1, cyclin D2, cyclin-dependent kinase 2 (CDK2), CDK4, phospho β-catenin, CK1α, GSK-3β, matrix metalloproteinase (MMP)-2, MMP-9, Akt, p-Akt and c-Myc were purchased from Cell Signaling Technology (Beverly, MA). The 3′–5′-cyclic adenosine monophosphate (cAMP) immunoassay kit was purchased from R & D System (Minneapolis, MN).

Cells and cell culture conditions. The human skin cancer cell lines, A431 and SCC13, were purchased from the American Type Culture Collection (Manassas, VA). They were cultured as monolayers in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA) as described in detail previously (Mantena et al., 2006). EGCG, dissolved in a small amount of PBS buffer; PGE_2, dissolved in dimethylsulfoxide (DMSO); and/or AH6809, dissolved in ethanol, were added to the complete cell culture medium [maximum concentration of ethanol or DMSO, 0.1% (v/v) in media] prior to addition of the media to sub-confluent cells (60–70% confluent). Cells treated with vehicle only served as a vehicle control. To determine the effect of EGCG on PGE_2-mediated effects, EGCG was added to the cell culture medium at least 30 min before the treatment of the cells with PGE_2.

MTT assay for cell viability. The effect of EGCG on cell viability was determined using the MTT assay, as described previously (Mantena et al., 2006). Briefly, 1×10^4 cells/well were plated in 96-well culture plates. After overnight incubation, the cells were treated with various concentrations of EGCG for 24, 48 and 72 h. The cells were then treated with 50 μl of 5 mg/ml MTT and the resulting formazan crystals were dissolved in DMSO (200 μl). Absorbance was recorded at 540 nm with a reference at 650 nm serving as the blank. The effect of EGCG on cell viability was assessed as percent cell viability compared to vehicle-treated control cells, which were arbitrarily assigned 100% viability. Cell viability was determined similarly after treatment of cells with PGE_2 or after transfection of cells with β-catenin or COX-2 siRNA.

Cell death analysis by trypan blue dye exclusion assay. To determine the effect of EGCG on cell death, the trypan blue dye exclusion assay was used, as described previously (Mantena et al., 2006). Briefly, 5×10^4 cells were cultured in each well of six-well culture plates. After overnight incubation, the cells were treated with various concentrations of EGCG (0, 10, 20, 40 and 60 μg/ml) for 24, 48 and 72 h. At the desired time points, the cells were harvested, treated with 0.25% trypan blue dye and the dead cells that had taken up the dye were counted under a microscope using a hemocytometer. The cytotoxic effects of EGCG are expressed as the mean ± SD percentage of dead cells in each treatment group. Each experiment was repeated twice.

Preparation of cell lysates and western blot analysis. Following treatment of cells for the indicated time periods, with or without EGCG, PGE_2 or AH6809, the cells were harvested and cell lysates prepared as described previously (Mantena et al., 2006; Vaid et al., 2011a, 2011b). Equal amounts of proteins were electrophoretically resolved on 10% tris–glycine gels and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with the primary antibody overnight at 4 °C. The membrane was then incubated with the appropriate peroxidase-conjugated secondary antibody and the immune-reactive bands visualized using enhanced chemiluminescence. To verify equal protein loading, the membrane was stripped and reprobed with anti-β-actin antibody.

cAMP immunoassay. Cells were treated with PGE_2, AH6809 and EGCG alone or in various combinations for 24 h at 37 °C. Thereafter, cells were washed with cold PBS and the levels of intracellular cAMP determined using a cAMP EIA kit following the manufacturer's protocol.

COX-2- or β-catenin-siRNA transfection of A431 and SCC13 cells. Human-specific COX-2-siRNA or β-catenin-siRNA was transfected into A431 and SCC13 cells using the siRNA transfection reagent kit (Santa Cruz Biotechnology, Inc.) following the manufacturer's protocol. Briefly, 2×10^5 cells/well were seeded in a 6-well plate and allowed to grow to 60–70% confluency. The COX-2 or β-catenin siRNA mix with transfection reagents were overlaid on the cells for approximately 6 h at 37 °C and the cells then transferred into 2× growth medium for about 18–20 h. At 24 h post-transfection, fresh medium was added to the cells and the cells were incubated for an additional 48 h. Thereafter, cells were harvested, lysates prepared and subjected to western blot analysis.

Statistical analysis. For the data analysis of cell viability, the data were compared between control and treated groups using one-way analysis of variance (ANOVA) using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. In each case $P < 0.05$ was considered statistically significant.

Results

EGCG inhibits proliferation/cell viability and induces cell death of human skin cancer cells

We first determined the effects of EGCG on the viability of the human skin cancer cell lines, A431 and SCC13. The cells were treated with 0, 10, 20, 40 and 60 μg/ml of EGCG for 24, 48 and 72 h. In both cell lines, EGCG treatment resulted in a significant reduction in cell proliferation/viability in a dose-dependent manner, as assessed using an MTT assay. Depending on the dose of EGCG, the reduction in viability of A431 cells ranged from 2.0 to 44% ($p < 0.05$) after 24 h, 4 to 53% ($p < 0.05$–0.01) after 48 h and 7 to 60% ($p < 0.05$–0.001) after 72 h of treatment (Fig. 1A). Similar cytotoxic effects were observed on treatment of SCC13 cells with EGCG (Fig. 1A, lower panel). In contrast, the MTT assay data suggested that EGCG is not toxic to normal skin cells if the cells were treated with EGCG at the dose of 10–60 μg/ml for 24 h. If cells are treated with EGCG (10, 20, 40 and 60 μg/ml) for 48 and