Epigallocatechin-3-gallate and trichostatin A synergistically inhibit human lymphoma cell proliferation through epigenetic modification of p16INK4a

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Abstract. DNA methylation and histone deacetylation play important roles in the occurrence and development of cancers by inactivating the expression of tumor suppressors, including p16INK4a, a cyclin-dependent kinase inhibitor. The present study investigated the effect of epigallocatechin-3-gallate (EGCG) alone or in combination with trichostatin A (TSA) on p16INK4a gene expression and growth in human malignant lymphoma CA46 cells. CA46 cell viability and cell cycle were analyzed; methylation of the p16INK4a gene was assessed by nested methylation-specific PCR (n-MSP). p16INK4a mRNA and protein expression was determined by real-time quantitative PCR and western blot analyses, respectively. Both EGCG and TSA alone inhibited CA46 cell proliferation; the combined treatment (6 µg/ml EGCG and 15 ng/ml TSA) significantly reduced CA46 cell proliferation from 24 to 96 h (all P<0.001). Cells treated with 24 µg/ml EGCG or the combination treatment (6 µg/ml EGCG and 15 ng/ml TSA) had lower proliferative indices when compared to the other groups. Co-treatment with EGCG and TSA decreased p16INK4a gene methylation, which coincided with increased p16INK4a mRNA and protein expression. Thus, EGCG and TSA synergistically reactivated p16INK4a gene expression in part through reducing promoter methylation, which may decrease CA46 cell proliferation.

Introduction

Cell cycle progression is regulated by a series of cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). p16INK4a, a member of the INK4 family of CDKIs and a tumor suppressor, inhibits CDK4, maintaining the hyper-phosphorylation of retinoblastoma (Rb) that suppresses cell cycle progression (1). The p16-cyclin D1-CDK4-Rb pathway regulates cell cycle transition from the G1 to the S phase; point mutations and/or epigenetic modifications in this pathway are observed in almost all human cancers (2,3). In addition to regulating the CDK4-Rb pathway, p16INK4a decreases eukaryotic elongation factor (eEF) 1A2 expression, reducing cancer elongation (4). It also induces cellular senescence in a cooperative manner with p21Waf1/Cip1, which was confirmed in double-knockout mice that were more susceptible to skin tumor formation (5). Moreover, enforced expression of p16INK4a in cisplatin-resistant non-small cell lung cancer cells increased their sensitivity to low cisplatin concentrations (6). Thus, treatments that increase p16INK4a expression may be beneficial in reducing cancer cell growth.

Epigenetic modifications, such as the methylation of CpG islands within gene promoters and histone deacetylation, can inactivate tumor-suppressor genes, including p16INK4a. Specifically, epigenetic regulation of p16INK4a expression through DNA methylation and/or histone deacetylation has been observed in many types of human cancers (2). Methylation of p16INK4a may be an early event in carcinogenesis (7). Furthermore, the detection of p16INK4a hypermethylation at higher concentrations in the plasma of breast cancer patients than in healthy control patients suggests that it may be a suitable tool for cancer screening, and its prognostic value has been suggested for breast and prostate cancer patients (7,8).

Epigallocatechin-3-gallate (EGCG), a green tea-derived polyphenol, was found to reduce DNA methylation of the p16INK4a promoter, reactivating its expression in the human pancreatic carcinoma cell line, PANC-1 and in human skin cancer cells (9,10). In addition, trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, elevated histone deacetylation of the HBP1 region, thereby inducing p16INK4a expression and subsequent cell apoptosis and differentiation (11). Thus, DNA methyltransferases (DNMTs) and HDACs may represent important therapeutic targets for the design of antitumor drugs (12,13).

The present study sought to prove the hypothesis that EGCG and TSA may synergistically enhance p16INK4a expression via epigenetic modification. After treatment with EGCG and TSA alone or in combination, cell proliferation, cell cycle

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progression, promoter methylation, and p16\textsuperscript{INK4a} expression were examined in human Burkitt lymphoma CA46 cells.

**Materials and methods**

Reagent preparation and cell culture. EGCG (Sigma, St. Louis, MO, USA) was diluted to 10 mg/ml with phosphate-buffered saline (PBS) (pH 7.4). TSA (Sigma) was diluted to 2 mg/ml with dimethyl sulfoxide (DMSO). Both were stored at -20°C until further use. The CA46 human Burkitt lymphoma cell line (CRL-1648™, ATCC, Atlanta, GA, USA) was maintained in RPMI-1640 complete medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Sijiqing, Zhejiang, China) at 37°C with 5% CO\textsubscript{2}.

Cell proliferation analysis. Cell proliferation was assessed with the MTT assay (Sigma) following the manufacturer’s instructions. Cells (4×10\textsuperscript{4}well) were seeded onto 96-well plates in 90 µl of growth medium containing the indicated treatment. On days 1, 2, 3 and 4, 10 µl of MTT solution was added. After 2-3 h at 37°C, optical density (OD) was measured at 450 nm, and the inhibition rate was determined as follows: \[ \frac{(OD_{\text{blank}} - OD_{\text{treatment}})}{OD_{\text{negative}} - OD_{\text{blank}}} \times 100\% \]. This experiment was performed three times.

Cell cycle analysis. CA46 cells (1×10\textsuperscript{6}well) were cultured with the indicated treatment for 48 h after which the cells were collected and washed twice with PBS. After fixing with 70% ethanol at 4°C overnight, the cells were stained with 40 µg/ml propidium iodide (PI; Sigma). Flow cytometry was performed with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). WinList v6.0 software (Topsham, ME, USA) was used to set the gate, and data were analyzed with ModFit LT V3.0 (Invitrogen). The proliferative index was determined as follows, where the G1, S, G2, and M values represent the proportion of cells in the corresponding phase: \( (S + G_2/M)/(G_1 + S + G_2/M) \times 100\% \). This experiment was performed three times.

Nested methylation-sensitive PCR (n-MSP). Genomic DNA was isolated from 2×10\textsuperscript{6} CA46 cells using the traditional chloroform extraction method followed by bisulfite modification using the EpiTect Bisulfite kit (Qiagen, Gaithersburg, MD, USA) following the manufacturer’s instructions and was stored at -20°C. n-MSP was performed with a two-stage nested approach as previously described by Palmisano et al (14) using the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Briefly, PCR amplification of a sequence of the p16 gene that was 280 bp in length and included a promoter region rich in CpG was undertaken using primers specific for bisulfite modification (primers for the first stage; Table I) and a region rich in CpG was undertaken using primers specific for the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used to set the gate, and data were analyzed with ModFit LT V3.0 (Invitrogen). The proliferative index was determined as follows, where the G1, S, G2, and M values represent the proportion of cells in the corresponding phase: \( (S + G_2/M)/(G_1 + S + G_2/M) \times 100\% \). This experiment was performed three times.

Western blot analysis. Cells (2×10\textsuperscript{6}) were collected after the indicated treatments for 72 h and lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was determined with a BCA kit (Pierce, Rockford, IL, USA) after which 60 µg was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After the membrane was blocked in non-fat milk at room temperature for 2 h, it was incubated with primary antibodies specific for p16 or β-actin (Abcam®, Hong Kong) at 4°C overnight. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce) at room temperature for 2 h. Visualization was carried out using the ECL Plus kit (Pierce).

Statistical analysis. The continuous variables are presented as the mean and standard deviation. For comparisons of the experimental groups, one-way ANOVA was used. When a significant difference between groups was apparent, multiple comparisons were performed using Tukey’s post-hoc test. The categorical variables were assessed using Pearson’s chi-square test. All statistical analyses were carried out using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

### Table I. Primer sequences for n-MSP and real-time PCR.

<table>
<thead>
<tr>
<th>Primer sequence (5’-3’)</th>
<th>n-MSP</th>
<th>p16 for first stage</th>
<th>p16 for methylated</th>
<th>p16 for unmethylated</th>
<th>q-PCR</th>
<th>p16</th>
<th>GAPDH</th>
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<tr>
<td>F: GAAGAAAGGAGGGTTGGG</td>
<td>R: CTACACACCTCTACCCACC</td>
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<tr>
<td>F: TTATTAGGGTGCCGATCGC</td>
<td>R: GACCGGGACCGGACGTA</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F: TTATTAGGGTGGGTGAATGT</td>
<td>R: CAACCCCCAACCAACCAA</td>
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</tr>
<tr>
<td>F: GAAATGGGATCAGGTAGC</td>
<td>R: GAGGAGGGTCTGTGATTAC</td>
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<td></td>
<td></td>
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<tr>
<td>F: GAAGGGTGAAGGCCGAGTCAAC</td>
<td>R: CAGAGTAAAAGCAGCCCTGGT</td>
<td></td>
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F, forward, R, reverse.
comparisons were performed using the Bonferroni procedure with type-I error adjustment. SAS software package, version 9.2 (SAS Institute, Inc., Cary, NC, USA) was used for the statistical analysis. All statistic assessments were evaluated at a two-sided $\alpha$ level of 0.05.

**Results**

**Effects of EGCC and TSA on CA46 cell growth.** As shown in Fig. 1, both EGCG and TSA inhibited CA46 cell proliferation after 48 h. Increasing EGCG concentrations did not further inhibit CA46 cell growth when compared to the growth of cells treated with 6 µg/ml EGCG (Fig. 1A), while significant inhibition was observed with 24 and 48 ng/ml TSA at 48 h when compared to the cells treated with 3 ng/ml TSA (both $P<0.001$; Fig. 1B). The effects of EGCG and TSA co-treatment on CA46 cell proliferation was assessed using various concentrations of each. Significantly greater growth inhibition was observed with 12 µg/ml EGCG + 30 ng/ml TSA, 6 µg/ml EGCG + 15 ng/ml TSA and 3 µg/ml EGCG + 7.5 ng/ml TSA when compared to cells treated with 1.5 µg/ml EGCG + 3.75 ng/ml TSA (all $P<0.001$; Fig. 1C).

Analysis of the effects of EGCG and TSA alone or in combination on CA46 proliferation over time was also assessed (Fig. 1D). From 24 to 96 h, CA46 cell survival was significantly higher in the untreated group when compared to cell survival in all of the treatment groups (all $P<0.001$). Furthermore, significantly reduced CA46 cell growth was observed in the combined treatment group (6 µg/ml EGCG and 15 ng/ml TSA) at 24 h and the effect continued until the end of the study (all $P<0.001$; Fig. 1D).

**Effects of EGCC and TSA on CA46 cell cycle progression.** Flow cytometry was performed to observe the effects of EGCG and TSA alone and in combination on cell cycle progression. With the exception of CA46 cells treated with 6 µg/ml EGCG, the proportion of cells in the G0/G1 phase was significantly higher in the treatment groups when compared to the proportion of cells in the control group (all $P<0.002$; Fig. 2A). Among the groups tested, the cells treated with 24 µg/ml EGCG and the combined treatment had the greatest proportion of cells in the G0/G1 phase. In contrast, with the exception of CA46 cells treated with 6 µg/ml EGCG, the proportion of cells in the S phase was significantly lower in the treatment groups than in the control group (all $P<0.001$; Fig. 2A). Analysis of the proliferative index revealed that the cells treated with 12 µg/ml EGCG, 24 µg/ml EGCG, 30 ng/ml TSA or the combination treatment had lower proliferative indices than the control group (Fig. 2B).
Effects of EGCG and TSA on the methylation of the p16\(^{\text{INK4a}}\) gene in CA46 cells. As shown in Fig. 3A, n-MSP analysis revealed a 151-bp band, corresponding to a methylated product, in the untreated controls, which decreased with increasing concentrations of EGCG. A corresponding increase in the 150-bp unmethylated product was observed with EGCG treatment at increasing concentrations (Fig. 3B). Whereas TSA did not dramatically alter the methylation status of the p16\(^{\text{INK4a}}\) gene, the combination treatment (6 µg/ml EGCG and 15 ng/ml TSA) decreased p16\(^{\text{INK4a}}\) methylation and concurrently increased the unmethylated status of the p16\(^{\text{INK4a}}\) gene to a greater degree than the untreated control (Fig. 3C and D).

Effects of EGCG and TSA on p16\(^{\text{INK4a}}\) expression. The effects of EGCG and TSA on p16\(^{\text{INK4a}}\) mRNA and protein expression were determined by RT-PCR and western blot analyses, respectively. As shown in Fig. 4A, p16\(^{\text{INK4a}}\) mRNA expression significantly increased with 24 µg/ml EGCG, 30 ng/ml TSA, and the combined treatment when compared to p16\(^{\text{INK4a}}\) mRNA expression in the control group (all P≤0.003). Notably, p16\(^{\text{INK4a}}\) mRNA expression in the combined treatment group was significantly higher than that of the other groups (all P<0.001; Fig. 4A). Similar results were observed for p16 protein expression. Both EGCG and TSA increased p16 protein expression in a dose-dependent manner (Fig. 4B and C). Furthermore, a combination of EGCG and TSA increased p16 expression to a
INK4a-INK4a expression may also mediate cell senescence. Methylation in the present study was not determined, we speculate that it may directly inhibit DNMT activity, inducing reactivation of methylation-silenced genes as previously reported (10,31-34). Direct interaction of EGCG to the catalytic site of DNMT 1 has been previously reported (19). These effects may be mediated at least in part through increased p16INK4a expression, which inhibits cancer cell proliferation and induces cellular senescence (4,5). Reduced p16INK4a expression may result in sustained binding of CDK4/CDK6 to cyclin D and subsequent phosphorylation of Rb protein, resulting in uncontrolled cell proliferation (2,3). p16INK4a expression may also mediate cell senescence (5,20); it may also inhibit cell growth by directly interacting with the eukaryotic elongation factor 1A2 (eEF1A2), reducing its expression (4). In addition, EGCG-induced apoptosis of Jurkat cells through hydrogen peroxide production has been demonstrated (21). Further analyses may determine whether the anti-proliferative effects of EGCG and TSA combination therapy are mediated by altered p16INK4a expression.

DNMT inhibitors not only interfere with the binding of methylation-sensitive transcription factors to target genes and directly inhibit RNA polymerase activity, but also indirectly alter histone acetylation (10). The methyl-CpG-binding protein (MeCP) can specifically bind to methylated DNA and recruit HDACs, thereby inducing focal histone deacetylation and transcription inhibition (22-24). Furthermore, TSA can reactivate the expression of genes which were reduced as a result of DNA methylation (25,26). In AML1/ETO-positive leukemia cells, the methyltransferase inhibitor, 5-azacytidine, increased the activity of FR901228, a histone deacetylase inhibitor, to elevate histone acetylation (27). In the present study, co-treatment with EGCG and TSA increased DNA demethylation of the p16INK4a gene to a greater extent than EGCG alone, which upregulated its expression. These results are similar to a study with leukemia cell lines (28), in which TSA and 5-aza-2′-deoxycytidine co-treatment reactivated the expression of genes with high methylation and static transcription; however, similar changes were not observed after treatment with TSA alone. Similarly, in the present study, TSA treatment alone did not dramatically alter the methylation status of the p16INK4a gene, suggesting that chromatin is maintained in a closed formation through DNA methylation, thus impairing the effect of TSA. In human skin cancer cells, EGCG not only inhibited DNA methylation but also increased histone acetylation (10). Therefore, further studies are necessary to analyze the levels of histone acetylation following EGCG treatment with and without TSA.

The mechanism underlying the EGCG-induced DNA demethylation is largely unclear. For example, it may inhibit dihydrofolate reductase and subsequent folate metabolism, thereby inhibiting DNA and RNA synthesis and reversing p16INK4a methylation (29,30). Conversely, EGCG may directly inhibit DNMT activity, inducing reactivation of methylation-silenced genes as previously reported (10,31-34). Direct interaction of EGCG to the catalytic site of DNMT 1 has been reported (32), in addition to oxidation of DNMT by EGCG (20). Furthermore, inhibition of HDACs by EGCG has been reported (21). Although the mechanism by which EGCG altered p16INK4a methylation in the present study was not determined, we speculate that it may directly inhibit DNMT activity or indirectly reduce their expression via interfering with folate metabolism. Further studies will be undertaken to test these postulations.
Reactivation of p16INK4a will likely be of clinical benefit to cancer patients as its expression has been associated with longer disease-free survival and better prognosis in human breast cancer (35). In addition, the prognostic value of determining p16INK4a promoter methylation has been reported for breast (7) and prostate cancers (8). Further in vivo studies are needed to evaluate the possible benefits of combining EGCG with a clinically useful HDAC inhibitor, such as suberoylanilide hydroxamic acid.

The present study is limited in that the mechanisms by which EGCG and TSA co-treatment reduced CA46 cell proliferation and reversed p16INK4a promoter methylation were not assessed. Further studies will be conducted to analyze HDAC and DNMT activity and histone acetylation after treatment with TSA and EGCG. In addition, the IC50 values of EGCG and TSA were not determined in the present study and, therefore, will be undertaken in further analyses.

Taken together, the additive effect of EGCG and TSA co-treatment on the increase in the expression of p16INK4a tumor suppressor and the inhibition of CA46 cell proliferation suggests that combination treatment containing HDAC and DNMT inhibitors may be useful in cancer treatment. Further in vivo study will be undertaken to fully elucidate the benefits of epigenetic modifications using EGCG with an HDAC inhibitor, such as suberoylanilide hydroxamic acid.

Acknowledgements

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References


