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EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS

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Abstract. Chronic inflammation raises the risk of the development of colorectal cancer (CRC). Green tea catechins (GTCs), which possess anti-inflammatory properties, are known to be anti-carcinogenic in a variety of organ sites, including the colorectum. This study investigated whether (-)-epigallocatechin gallate (EGCG), a candidate chemopreventive agent and major biologically-active component of green tea, and Polyphenon E (Poly E), a mixture of GTCs, suppress inflammation-related colon carcinogenesis induced by azoxymethane (AOM) and dextran sodium sulfate (DSS). Male ICR mice aged 5 weeks were given a single intraperitoneal injection of AOM (10 mg/kg body weight), followed by 2% (w/v) DSS in drinking water for 7 days to induce colitis-related colonic tumors. They also received drinking water containing EGCG (0.01 or 0.1%) or Poly E (0.01 or 0.1%) up to week 17. At week 17, treatment with EGCG or Poly E had significantly suppressed the multiplicity and volume of colonic neoplasms as compared to the AOM/DSS group, and had resulted in a lesser degree of malignancy. In addition, treatment with EGCG or Poly E decreased the protein and mRNA expression levels of Cyclooxygenase (COX)-2 and the mRNA expression of inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12 and IL-18) in the colonic mucosa. Our findings provide evidence that tea catechins are beneficial to the suppression of cancer development in the inflamed colon.

Introduction

Colorectal cancer (CRC) is a major health care problem worldwide. Several lines of evidence indicate that chronic inflammation is a key predisposing factor to the development of CRC (1). It is one of the most serious complications in inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease (2,3). Patients with IBD are therefore a group at high risk for CRC, and need careful follow-up (4). The clinical characteristics of the disease also suggest that patients with IBD are appropriate targets for the chemoprevention of CRC, and its primary prevention in IBD has been receiving a lot of attention of late. In clinical studies, 5-aminosalicylic acid (ASA), an anti-inflammatory drug, has been shown to protect against the development of CRC in IBD patients (5,6). This suggests that the agents targeting inflammation-associated molecules are able to inhibit the development of IBD-related CRC. Recent studies have indicated that certain types of phytochemicals, including green tea catechins (GTCs), possess anti-inflammatory and anti-oxidant properties, and that these effects are responsible for their cancer chemopreventive potency (7,8). Many experimental studies with rodents have shown that green tea or its constituents can inhibit either carcinogenesis or the growth of chemically-induced cancers in various tissues, including the colorectum (7). (-)-Epigallocatechin gallate (EGCG), one of the major constituents of green tea, is the most potent polyphenolic compound in green tea with respect to inhibiting proliferation and inducing apoptosis in cancer cells (9-11). Our previous studies have demonstrated that EGCG inhibits the growth of human CRC cells and induces apoptosis in cancer cells (12-14). In addition, EGCG can inhibit the expression of cyclooxygenase (COX)-2, one of the main mediators in the inflammatory signaling pathway, by inhibiting the activation of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) in SW837 CRC cells (13).
These findings are important because increased COX-2 expression plays a significant role in CRC development, and might therefore be one of the targets of CRC chemoprevention (15,16).

COX-2 expression in tissues is induced by several pro-inflammatory gene products, as well as by RTKs (17). Among the molecules involved in inflammation, tumor necrosis factor (TNF-α), a central mediator in chronic inflammatory diseases, plays a critical role in the development of epithelial malignancies. This is based on the finding that TNF-α stimulates tumor promotion and progression in carcinogenesis (18-20). Moreover, EGCG or green tea extracts inhibit activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) activities and decrease the expression level of inflammatory mediator genes, including the TNF-α gene, thus suppressing the growth of cancer cells (19,20). EGCG may therefore prevent chronic inflammation of the bowel and inhibit the development of CRC in the inflamed colon. To confirm this hypothesis, the potential chemopreventive ability of EGCG and Polyphenon E (Poly E), a decaffeinated extract of green tea that contains 60% EGCG and lesser amounts of other tea catechins (12), was examined in a colitis-related mouse CRC model induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) (21,22). The focus was on the expression levels of COX-2 and other important inflammatory mediators, such as TNF-α, interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-12 and IL-18.

Materials and methods

Animals, chemicals and diets. Male ICR mice aged 5 weeks (Charles River Japan Inc., Tokyo, Japan) were maintained at the Gifu University Animal Facility according to its Institutional Animal Care Guidelines. All mice were housed in plastic cages with free access to drinking water (tap water with or without EGCG or Poly E) and a pelletted basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle) and temperature (23±2˚C). They were quarantined for the first 7 days, and then separated randomly according to body weight into experimental and control groups. AOM, a colonic carcinogen, was purchased from Sigma Chemical Co. (St. Louis, MO). DSS was purchased from MP Biomedicals, LLC (Aurora, OH). DSS for the induction of colitis was dissolved in distilled water at a concentration of 2% (w/v). EGCG and Poly E were kindly provided by Mitsui Norin Co., Ltd. (Tokyo, Japan). Poly E contains about 60% EGCG, 7% EC, 12% EGC, 1% ECG and 2% GCG (12). A freshly prepared solution of EGCG or Poly E in tap water was supplied every Monday, Wednesday and Friday to the experimental animals.

Experimental procedure. A total of 65 male ICR mice were divided into 8 (experimental and control) groups (Fig. 1). The mice in Group 1 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting from 1 week after the injection, the mice received 2% DSS in their drinking water for 7 days, and thereafter no further treatment up until week 17. Groups 1-5 were treated with AOM and 2% DSS, then tap water containing 0.01% EGCG, 0.1% EGCG, 0.01% Poly E and 0.1% Poly E was given ad libitum to Groups 2-5, respectively, for 14 weeks, starting 1 week after the cessation of DSS exposure. Group 6 was given just 0.1% EGCG and Group 7 just 0.1% Poly E, while Group 8 served as an untreated control. During the study, body weight, intake of drinking water with or without DSS and diet was recorded every day. All mice were sacrificed at week 17 and the major organs (liver, spleen and kidney) were weighed. At the time of sacrifice, large bowels were excised and washed with phosphate-buffered saline (PBS). After measuring their length (from the ileocecal junction to the anal verge), they were cut open longitudinally along the main axis then flushed with PBS. The large intestines were macroscopically inspected and the volume of tumors, if present, was measured. Tumor volume was calculated using the equation V = 4/3πr³, with r as the average tumor radius obtained from three diameter measures. The colons were then cut and fixed in 10% buffered formalin for 24 h. Histopathological examination of the large intestine, liver, kidney and spleen was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. After resection of the colonic neoplasms, the mucosa without tumors was scraped by surgical knife for the extraction of proteins and RNAs.

Scoring of inflammation in the large bowel. Inflammatory scores in the large bowel were determined using the inflammation scoring system, as described previously (23). Large intestinal inflammation on the H&E sections was graded
**Table I. Body, liver, spleen and kidneys weights and lengths of the large bowel of mice.**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>Body weight (g)</th>
<th>Relative organ weight (g/100 g body weight)</th>
<th>Length of large bowel (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>1</td>
<td>AOM/DSS</td>
<td>10</td>
<td>40.1±3.1a</td>
<td>5.62±0.37</td>
<td>0.85±0.50</td>
</tr>
<tr>
<td>2</td>
<td>AOM/DSS/0.01% EGCG</td>
<td>10</td>
<td>42.9±4.5</td>
<td>5.00±0.35b</td>
<td>0.43±0.16b</td>
</tr>
<tr>
<td>3</td>
<td>AOM/DSS/0.1% EGCG</td>
<td>10</td>
<td>44.3±3.9</td>
<td>4.95±0.32b</td>
<td>0.42±0.09b</td>
</tr>
<tr>
<td>4</td>
<td>AOM/DSS/0.01% Poly E</td>
<td>10</td>
<td>40.8±3.0</td>
<td>4.98±0.47b</td>
<td>0.48±0.34</td>
</tr>
<tr>
<td>5</td>
<td>AOM/DSS/0.1% Poly E</td>
<td>10</td>
<td>40.2±3.1</td>
<td>5.26±0.37</td>
<td>0.57±0.32</td>
</tr>
<tr>
<td>6</td>
<td>0.1% EGCG</td>
<td>5</td>
<td>40.3±3.0</td>
<td>5.40±0.31</td>
<td>0.43±0.05</td>
</tr>
<tr>
<td>7</td>
<td>0.1% Poly E</td>
<td>5</td>
<td>42.1±1.4</td>
<td>4.82±0.16</td>
<td>0.32±0.08</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>5</td>
<td>43.3±2.7</td>
<td>5.07±0.41</td>
<td>0.40±0.09</td>
</tr>
</tbody>
</table>

aMean ± SD. bSignificantly different from Group 1 (p<0.01). cSignificantly different from Group 1 (p<0.05). dSignificantly different from Group 8 (p<0.05).

According to the morphological criteria of Cooper et al (24). Scoring was made on the entire colon and the findings were expressed as the average of each score per mouse.

**Protein extraction and Western blot analysis.** Total proteins were extracted from the scraped colonic mucosa of AOM/DSS-treated mice (Groups 1-5), and equivalent amounts of proteins (40 μg/lane) were examined by Western blot analysis, as described previously (13). The primary antibodies for COX-2 (SC7951) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (SC7724) proteins were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-mouse or anti-rabbit IgG antibodies (GE Healthcare, Buckinghamshire, UK) were used as the secondary antibodies. Each membrane was developed using an enhanced chemiluminescence detection system (GE Healthcare). An antibody to GAPDH served as the loading control. The intensities of the blots were quantified with NIH Image software version 1.61. Each mRNA level was expressed as its ratio to GAPDH protein, and was then analyzed statistically.

**RNA extraction and semi-quantitative RT-PCR analysis.** RNA extraction and semi-quantitative RT-PCR analysis were also performed as described previously (13,14). Total-RNA was isolated from the scraped colonic mucosa of the AOM/DSS-treated mice (Groups 1-5) using Isogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. The cDNA was amplified from 1 μg of total-RNA using SuperScript one-step RT-PCR with the platinum Taq system (Invitrogen, Carlsbad, CA). The primers used for amplification of the COX-2, TNF-α, IFN-γ, IL-6, IL-12, IL-18 and GAPDH specific genes were as follows: COX-2 forward 5′-AAG CCT TCT CCA ACC TCT CC-3′, reverse 5′-GGT TGG GCC ATA GAA CT-3′; TNF-α forward 5′-CAT GCG TCC TCG AGC TGA CTA AA-3′, reverse 5′-AGG GTG TGG GCC GTA AAA CT-3′; IFN-γ forward 5′-GCC CAT CAG CAA AAA AG-3′, reverse 5′-CGC AAT CAC AGT CTT GGC TA-3′; IL-6 forward 5′-GTT TTC TGG AGG GAA ATC GTG GA-3′, reverse 5′-CGC ACT AGG TTT GCC GAG TA-3′; IL-12 forward 5′-ACG GCC AGA AAA CTG AA-3′, reverse 5′-CAG ATA GCC CAT CAC CCT GT-3′; IL-18 forward 5′-GCC TCA AAC CTT CCA AAT CA-3′, reverse 5′-TGG ATC CAT TTC CTC AAA GG-3′; GAPDH forward 5′-AGC TTG TTA CTA TCA ACA AGG-3′, reverse 5′-GGA TGC AGG GAT GAT CT-3′. Using a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA), 35-cycle rounds of PCR were chosen for data analysis for the expression of these mRNAs, since a semi-quantitative assessment indicated that the reaction had not reached a plateau and was still in the log phase. The amplified products obtained with GAPDH-specific primers served as an internal control. The intensities of the PCR products separated on an agarose gel and stained with ethidium bromide were quantified with NIH Image software version 1.61. Each mRNA level was expressed as its ratio to GAPDH mRNA, then analyzed statistically.

**Statistical analysis.** The data, with the exception of the incidence of lesions, were expressed as the mean ± SD. Statistical significance was tested using GraphPad InStat software version 3.05 (GraphPad Software Inc., San Diego, CA). Tumor incidence was analyzed using Fisher's exact probability test or the χ² test, and other data were analyzed using the Student's t-test and the one-way ANOVA with Bonferroni's correction. P-values <0.05 were considered statistically significant.

**Results**

**General observation.** Bloody stool was observed in the mice that had received AOM plus DSS (Group 1). In a few Group 1 mice, anal prolaps due to tumor development in the distal colon was observed as well. The body weight, main organ (liver, spleen and kidneys) weight and the length of the large bowels of the mice in all groups at the end of the study (week 17) are listed in Table I. There were no significant differences in the mean body weight of the groups. The relative liver weight of the mice in Groups 2 (AOM/DSS/0.01% EGCG), 3 (AOM/DSS/0.1% EGCG) and 4 (AOM/DSS/0.01% Poly E) was significantly lighter than in Group 1 (p<0.01), as was the relative spleen weight of the mice in Groups 2 and 3 (p<0.05).
However, there were no pathological alterations suggesting toxicity of the GTCs in the liver, kidneys and spleen of mice (data not shown). The mean length of the large bowel in the Group 1 mice was significantly shorter than that of the untreated group (p<0.05).

Table II. Incidence, multiplicity and volume of colonic neoplasms.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>Incidence (%)</th>
<th>Multiplicity (no. of neoplasms/mouse)</th>
<th>Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AOM/DSS</td>
<td>10</td>
<td>10/10 (100)</td>
<td>15.45±8.55­ 7.20±4.64 8.25±4.76</td>
<td>44.2±44.5</td>
</tr>
<tr>
<td>2</td>
<td>AOM/DSS/0.01% EGCG</td>
<td>10</td>
<td>9/10 (90)</td>
<td>8.78±4.83 5.79±3.71 2.99±3.61­</td>
<td>30.2±34.2</td>
</tr>
<tr>
<td>3</td>
<td>AOM/DSS/0.1% EGCG</td>
<td>10</td>
<td>9/10 (90)</td>
<td>10.53±6.62 5.44±3.83 5.09±3.46</td>
<td>35.0±29.6</td>
</tr>
<tr>
<td>4</td>
<td>AOM/DSS/0.01% Poly E</td>
<td>10</td>
<td>9/10 (90)</td>
<td>9.31±6.41 5.62±3.68 3.69±2.92­</td>
<td>27.8±26.1</td>
</tr>
<tr>
<td>5</td>
<td>AOM/DSS/0.1% Poly E</td>
<td>10</td>
<td>9/10 (90)</td>
<td>7.55±3.89 4.39±2.65 3.16±2.31­</td>
<td>23.1±22.7b</td>
</tr>
<tr>
<td>6</td>
<td>0.1% EGCG</td>
<td>5</td>
<td>0/5 (0)</td>
<td>0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.1% Poly E</td>
<td>5</td>
<td>0/5 (0)</td>
<td>0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>5</td>
<td>0/5 (0)</td>
<td>0 0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

aMean ± SD. bSignificantly different from Group 1 (p<0.01). cSignificantly different from Group 1 (p<0.05).

They were histologically-tubular adenoma (Fig. 2a) or well/moderately-differentiated tubular adenocarcinoma (Fig. 2b) with infiltration in the submucosa (Fig. 2c). Dysplasia (Fig. 2d) also developed in the mice of these groups. There were no proliferative lesions on the colon of mice in Group 6 (EGCG alone) or 7 (Poly E alone). Table II summarizes the incidence, multiplicity and volume of colonic neoplasms. The incidence (100 or 90%) of colonic tumors did not significantly differ in Groups 1-5. However, the multiplicity of colonic adenocarcinoma in Groups 2, 4 and 5 was significantly lower

Figure 2. Histopathology of colonic lesions induced by AOM plus 2% DSS. (a) Tubular adenoma, (b and c) tubular adenocarcinoma and (d) dysplasia developed in a mouse from Group 1. (c) Indicates an invasion of tubular adenocarcinoma. H&E staining, original magnification, (a, c and d) x10, (b) x4.
than in Group 1 (p<0.05). The tumor volume of GTC-treated groups (2-5) was smaller than in Group 1. Only that of Group 5 was significantly different (p<0.01).

Effect of EGCG and Poly E on inflammation in the large bowel. Fig. 3A illustrates that the inflammation scores of Groups 2-5, which were given the EGCG- or Poly E-containing water, were significantly smaller than those of the GTC-untreated group (Group 1, p<0.05).

Effect of EGCG and Poly E on the expression levels of COX-2 proteins and mRNAs in AOM plus DSS-treated colonic mucosa. Treatment with EGCG (Groups 2 and 3) and Poly E (Groups 4 and 5) caused a marked decrease in the expression levels of both COX-2 proteins (Fig. 3B) and mRNAs (Fig. 3C) in the colonic mucosa in comparison to the GTC-untreated group (Group 1).

Effect of EGCG and Poly E on the expression levels of inflammatory cytokines in the colonic mucosa. We determined whether GTCs in drinking water modify the expression levels of proinflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-12 and IL-18 mRNAs, which play an important role in IBD (25-27). Semi-quantitative RT-PCR analyses revealed that EGCG and Poly E cause a significant decrease in the expression levels of these inflammatory cytokine mRNAs as compared to Group 1 (Fig. 4). This reduction was most apparent in mice treated with 0.1% EGCG (Fig. 4, column 3).

Discussion

The prevention of CRC by the administration of chemopreventive agents is a promising strategy for IBD patients who are at increased risk of epithelial malignancy. The results of our study clearly indicate that GTCs effectively inhibit the inflammation-related mouse colon carcinogenesis induced by AOM plus DSS (Table II). This inhibition is considered to be associated with the suppression of inflammation (Fig. 3A) and the inhibition of COX-2 expression (Fig. 3B and C). These findings are consistent with a previous report, which showed that green tea extracts in drinking water reduced COX-2 expression and suppressed the formation of preneoplastic colonic lesions induced by AOM in rats (28). EGCG also inhibits the expression of COX-2 and the biosynthesis of prostaglandin E₂ (PGE₂), a major product of the COX-2 enzyme, by inhibiting the activation of the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways in CRC cells (13,29). EGCG thus inhibits tumor growth and, at least in part, induces apoptosis by interfering with the COX-2/PGE₂ axis in colon cancer cells (13). In addition, overexpression of PGE₂ and its receptors is implicated in the pathophysiology of IBD and colorectal neoplasia (30).

We should emphasize that although Poly E, on a weight basis, contains only about 60% EGCG (12), it is equally as efficient as EGCG alone in preventing the development of IBD-related CRC (Table II) by attenuating inflammation in the colonic mucosa (Figs. 3 and 4). This might be associated with the presence of Poly E of epicatechin (EC), an inert tea catechin, because combined treatment with EGCG plus EC caused a synergistic inhibition of growth and the induction of apoptosis in cancer cells (12,31). It also should be stressed that not only a high (0.1%), but also a low (0.01%) concentration of EGCG and Poly E caused a decrease in the development of adenocarcinoma and reduced tumor volume (Table II) by inhibiting inflammation in the large bowel induced by AOM
plus DSS (Figs. 3 and 4). The lower dose is more acceptable for administration to patients.

The anomalous expression of COX-2 plays a critical role in the development of CRC; therefore, targeting this molecule might be an effective strategy for CRC chemoprevention (15,16). Using the present experimental model, Kohno et al (32) reported that COX-2 inhibitor suppresses colitis-related colonic carcinogenesis by inhibiting the expression of COX-2. In addition to the findings of the present in vivo study (Fig. 3B and C), EGCG caused a decrease in COX-2 expression in CRC cells (13). Although the precise mechanism how EGCG can inhibit the expression of COX-2 remains unclear, the inhibition of activation of RTKs by EGCG might play a role because the EGFR family of RTKs is implicated in the induction of COX-2 expression in CRC cells (33). A clinical study has suggested that the oral ingestion of green tea causes a rapid decrease in the level of PGE2 in the rectal mucosa (34). EGCG inhibits COX-dependent arachidonic acid metabolism in human colon mucosa and tumors (35). The expression of EGFR is common in IBD-related CRC, and might be a promising target for the chemoprevention of this type of malignancy (36). These findings, together with those of in vitro studies on colon cancer cells (12-14) and current in vivo studies, encourage clinical prevention studies with GTCs for CRC in IBD patients.

Among the specific cytokines that determine the pathological condition of IBD, TNF-α is considered an important tumor promoter in inflammation-related carcinogenesis (18-20). Activation of the transcription factor NF-κB, which is frequently found in the inflamed mucosa of IBD patients, is highly associated with the induction of TNF-α (37). Recent studies have indicated that EGCG or green tea extract reduces the expression of TNF-α by inhibiting AP-1 and NF-κB promoter activities (19,20). EGCG and Poly E also inhibit the transcription activities of the AP-1 and NF-κB promoters in CRC cells (12,13). The production of chemokines and PGE2 by TNF-α stimulation is inhibited by treatment with EGCG in human colon epithelial cells (38). EGCG also inhibits the expression of IL-6, the other key molecular in the pathogenesis of IBD (26), by attenuating NF-κB activity (20,39). Chronic inflammation influences the development of IBD-related colorectal carcinogenesis (1). Our finding that EGCG and Poly E decrease the expression levels of inflammatory cytokines, including TNF-α and IL-6, in the inflamed colon (Fig. 4) suggests the possibility that the inhibition of inflammation by green tea extracts is a potentially effective strategy for reducing the risk of CRC development in IBD patients.

In conclusion, the results of this experiment support the possibility that the inhibition of inflammation by GTCs may be a potentially effective and critical strategy for IBD-related CRC.

Figure 4. Effects of drinking EGCG and Poly E on the expression of inflammatory cytokine mRNAs in the colonic mucosa of mice treated with AOM plus 2% DSS. The scraped colonic mucosa obtained from AOM/DSS-treated mice (Groups 1-5) were examined by semiquantitative RT-PCR analysis using their respective primers (Table I). Amplified PCR products obtained with GAPDH specific primers served as internal controls. The results obtained from RT-PCR analysis were quantitated by densitometry and are displayed in the lower panels, respectively. Similar results were obtained in repeat experiments. Values are the mean ± SD. *p<0.05, **p<0.01: significant differences obtained by comparison with GTC-untreated mice (Group 1).
chemo prevention. Poly E may be preferable to EGC because it is easier to prepare and because the mixture of catechins it includes might exert a synergistic growth inhibitory effect.

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