Immunoprotective Effect of Epigallocatechin-3-gallate on Oral Anticancer Drug-Induced α-Defensin Reduction in Caco-2 Cells

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The aim of this study was to determine the effect of interaction between tegafur (FT) and epigallocatechin-3-gallate (EGCG) on the expression of α-defensins (HD-5: human α-defensin 5, HD-6: human α-defensin 6) by using a Caco-2 cell line as a model of human intestinal epithelial cells. This is the first study in which the effect of interaction of an oral anticancer drug and functional food on the innate immune system was examined. α-Defensins are abundant constituents of mouse and human paneth cells and play a role in the innate immune system in intestine. We detected HD-5 and HD-6 mRNA in Caco-2 cells and evaluated the effects of FT and EGCG on these mRNA levels. HD-5 and HD-6 mRNA levels were decreased by exposure to FT. Production of reactive oxygen species (ROS) was induced by exposure to FT as well as H2O2 exposure, and EGCG suppressed FT-induced production of ROS. Furthermore, FT-induced decrease in HD-5 and HD-6 mRNA levels was almost completely suppressed by EGCG. These results indicate that EGCG restored the decrease of α-defensins induced by FT at the transcriptional level in Caco-2 cells, suggesting that EGCG can be used as adjunctive therapy in chemotherapy.

Key words α-defensin; epigallocatechin-3-gallate; oral anticancer drug; innate immunity

Anticancer drugs can cause a decrease in innate immune response function in the intestine, leading to severe inflammation and diminished barrier function. Although tegafur (FT), which is the main ingredient of oral anticancer drugs, may give extreme functional decline to touch intestine directly, the effect of an anticancer drugs give on the intestinal tract has not been examined. Defensins are antimicrobial peptides secreted by various cells as components of innate immunity and are classified broadly into two groups, α- and β-defensins. α-Defensin (HD)-5 and -6 were antimicrobial peptides secreted as components of the innate host defense system as well as lysozyme and secreted phospholipase A2. β-Defensin (HD)-5 and HD-6 play an essential role for gut epithelia in regulating host bacteria at the mucosal surface. Although α-defensins are considered to be important in innate immunity in the intestine, have not been studied the relationships between intestinal immunity peptides and cancer diseases.

Supplements are being used by many cancer patients to alleviate side effects of chemotherapy and therapy-related symptoms such as immune compromise. Some nutraceuticals have been approved as dietary foods for special medical purposes for cancer patients. The interactions between supplements and anticancer drugs may increase or decrease the pharmacological or toxicological actions. Epigallocatechin-3-gallate (EGCG) is recognized as an effective functional food that has ecological or toxicological actions. Epigallocatechin-3-gallate (EGCG) are recognized as an effective functional food that has ecological or toxicological actions. Epigallocatechin-3-gallate (EGCG) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. FT and EGCG were dissolved in culture medium. All other reagents were of the highest grade available and used without further purification.

Cell Culture Caco-2 cells obtained from RIKEN (Ibaraki, Japan) were maintained in plastic culture flasks (Corning Coster Corp., Cambridge, MA, U.S.A.). The medium consisted of Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Japan, Tokyo) supplemented with 10% fetal bovine serum, 1% nonessential amino acid (Gibco, Grand Island, NY, U.S.A.), 2 mM L-glutamine and 100 IU/mL penicillin-100 µg/mL streptomycin (Sigma-Aldrich Japan, Tokyo). Monolayer cultures were grown in an atmosphere of 5% CO2 at 37°C. In the present study, Caco-2 cells were used between passages 46 and 50.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was extracted from the cell lysate using an Isogen according to the manufacturer’s instructions. Steady-state levels of HD-5, HD-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by real-time PCR. Total RNA (5 µg) was reverse-transcribed with an Omniscript RT Kit. The obtained cDNA (1 µg) was amplified by an SYBER Green real-time PCR kit using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). The reaction mixtures was incubated for 15 min at 95°C and then subjected to 40 amplification cycles consisting of denaturing at 94°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for 60 s. The

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The objective of this study was to determine the effect of FT on the expression of α-defensins and the effect of interaction between FT and EGCG on the expression of α-defensins by using a Caco-2 cell line as a model of human intestinal epithelial cells.

MATERIALS AND METHODS

Chemicals Tegafur (FT) and epigallocatechin-3-gallate (EGCG) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. FT and EGCG were dissolved in culture medium. All other reagents were of the highest grade available and used without further purification.

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primer sequences were as follows: HD-5, 5'-cgc cat cct tgc tgc cat tct-3' (forward) and 5'-aac ggc cgg ttc ggc aat agc-3' (reverse); HD-6, 5'-gtg ggg caa atg acc agg act-3' (forward) and 5'-tcc ctc aga ggc agc aga atc-3' (reverse), and GAPDH, 5'-aag gtc atc cct gag ctg aa-3' (forward) and 5'-ttc tag acg gca ggt cag gt-3' (reverse). The gene expression levels were finally normalized by using GAPDH as a housekeeping gene.

**Measurement of Intracellular Reactive Oxygen Species (ROS)** Cellular oxidative stress was measured using the cell-permeable indicator 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by cellular esterases to form nonfluorescent 2,7-dichlorodihydrofluorescein (DCFH) after penetrating into cells of test organisms, and then DCFH is immediately transformed to highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS, such as hydroxyl radical and H₂O₂-peroxidase, but not superoxide radical anion or H₂O₂ alone.⁷) The stock solution of DCFH-DA was prepared in ethanol at a concentration of 4 mM and stored at −30°C. The level of ROS was determined by the methods described by Schrader et al.⁸) Caco-2 cells were pretreated for 3 h with various concentrations of H₂O₂, EGCG or FT in the presence of 10 µM DCFH-DA at 37°C. Fluorescence was finally measured using a spectrometric microplate reader at 490 nm.

**Statistical Analyses** Student’s t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by the Tukey–Kramer test. A value of p<0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Effect of FT on HD-5 and HD-6 mRNA Levels and ROS Production in Caco-2 Cells** First, we examined the alterations in HD-5 and HD-6 mRNA levels induced by FT in Caco-2 cells. HD-5 and HD-6 mRNA levels were significantly reduced by FT at 3 h and had returned to pretreatment levels at 12 h (Figs. 1a, b). On the other hand, these concentrations or exposure times of FT had no effect on the viability of Caco-2 cells (Supplemental Fig. 1a). Anticancer drugs are known to induce production of ROS, ROS cause severe damage such as immune compromise. However, the possibility of induced production of ROS reducing HD-5 and HD-6 has not been investigated. So we examined the effects of H₂O₂ and FT on ROS production and HD-5 and HD-6 expression. Since exogenous H₂O₂ is known to diffuse into the cell nucleus and injure DNA by generating hydroxyl radical in culture cells,⁹) H₂O₂ was used as a positive control in this study. H₂O₂ and FT induced production of ROS in a concentration-dependent manner (Figs. 1c, d). Our results showed that 100 µM FT
Table 1. Effect of Epigallocatechin-3-gallate (EGCG) on FT-Induced Decrease in α-Defensin mRNA Expression in Caco-2 Cells

<table>
<thead>
<tr>
<th>Relative mRNA level (fold)</th>
<th>HD-5</th>
<th>HD-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>0.21±0.16*</td>
<td>0.59±0.17*</td>
</tr>
<tr>
<td>FT+EGCG1</td>
<td>0.81±0.24ns</td>
<td>0.63±0.29ns</td>
</tr>
<tr>
<td>FT+EGCG10</td>
<td>1.11±0.39ns</td>
<td>0.98±0.34ns</td>
</tr>
<tr>
<td>FT+EGCG100</td>
<td>1.06±0.34ns</td>
<td>1.29±0.85ns</td>
</tr>
</tbody>
</table>

Caco-2 cells were treated with 100 μM FT and 1, 10, 100 μM EGCG for 3 h. Each value (n=6–8) is the mean±S.D. of 3 independent experiments. *; significantly different from control at p<0.05; ns; not significant.

Fig. 2. Effects of EGCG on FT-Induced Production of ROS in Caco-2 Cells

Caco-2 cells were treated with FT (100 μM) and EGCG (100 μM) for 3 h. The bar graphs (n=6–8) are given as means with S.D. of more than two independent experiments. *; significantly different from control at p<0.05.

induced production of ROS to the same degree as did 1 mM H2O2. Moreover, to verify that the suppressive effect of FT on HD-5 and HD-6 expression was due to ROS, we examined the mechanism that EGCG increased the HD-5 mRNA level (Supplemental Fig. 1b).

Effect of EGCG on FT-Induced α-Defensin Reduction in Caco-2 Cells

Next, we further examined the effect of EGCG on the FT-induced reduction of HD-5 and HD-6 mRNA levels in Caco-2 cells. As shown in Table 1, EGCG restored the FT-induced decrease of HD-5 and HD-6 mRNA levels in a dose-dependent manner. To verify that this effect of EGCG on FT-induced decrease in HD-5 and HD-6 mRNA levels was due to suppression of ROS production, we examined the effect of EGCG on ROS production by exposure to FT. EGCG significantly suppressed the FT-induced ROS production and protected Caco-2 cells from oxidative stress (Fig. 2). And 100 μM EGCG increased HD-5 mRNA level (Supplemental Fig. 1b). The mechanism that EGCG increased the HD-5 mRNA level is unclear. It has been reported that EGCG activates the extracellular signal-regulated kinase (ERK) signaling pathway. On the other hand, other types of defensins are upregulated by the activation of this pathway. Collectively, we suggest that EGCG may increase HD-5 expression by ERK signaling pathway in Caco-2 cells. These results suggest that, on temporary damage by ROS of anticancer drug, antioxidative food may affect the improvement of innate immune system.

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