Inhibitory Activity of Catechin Metabolites Produced by Intestinal Microbiota on Proliferation of HeLa Cells

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Eleven kinds of catechin metabolites produced from (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) by intestinal microbiota were evaluated for inhibitory activity on the proliferation of HeLa cells, which are human cervical cancer cells. Among the catechin metabolites, 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M2), 4-hydroxy-5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M7), and 5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M9) were found to show inhibitory activity on HeLa cell proliferation as compared with control. The results suggested that three adjacent hydroxyl groups in the phenyl moiety may play an important role in the inhibitory activity. In addition, the inhibitory activity was also examined with four (−)-epicatechin (EC) metabolites possessing two adjacent hydroxyl groups in the phenyl moiety. Only 5-(3,4-dihydroxyphenyl)valeric acid (EC-M9) showed inhibitory activity and therefore valeric acid moieties likely contributes to the inhibitory activity. EGC-M9 showed the strongest inhibitory activity with IC₅₀ of 5.58±0.18. Thus, in this study it was found for the first time that several catechin metabolites derived from EGC, EGCg, and EC inhibit the proliferation of cervical cancer cells.

Key words epigallocatechin gallate; metabolite; cervical cancer; catechin; 5-(3,4,5-trihydroxyphenyl)valeric acid; HeLa cell

Tea catechins are natural constituents which have various physiological functions such as antioxidative,1,2) blood glucose-lowering,3) hypotensive,4,5) and anticancer activities.6) However, it has been reported that the absorption rate of a major tea catechin, (−)-epigallocatechin gallate (EGCG), was 0.1 to 1.6% of the oral dose in rats.7) We also estimated the bioavailability of EGCg including its conjugated forms to be 0.26% after oral dosage of [4-3H]EGCg in rats.8) In humans, the bioavailability of tea catechins has been reported to be less than 4%.9) Thus, intact tea catechins have been regarded as being poorly absorbed in the body. On the other hand, tea catechins are recognized to be metabolized by intestinal microbiota after reaching the colon.10–13) The metabolites such as 5-hydroxyphenyl−γ-valerolactones, 4-hydroxy-5-hydroxyphenyl valeric acids, and 3-hydroxyphenyl propionic acids were absorbed into the body and were detected in urine.14–18) Particularly, 5-hydroxyphenyl−γ-valerolactones were recognized to be dominant urinary metabolites in rats and human urine and their excretion amounts reached 6–39% of the ingested catechins.8,9,19)

Catechin metabolites have been reported to show bioactivities such as antioxidative, anti-inflammatory, anti-thrombotic, and blood pressure lowering activities.18,20,21) Kim et al. recently showed that 5-(3,5-dihydroxyphenyl)−γ-valerolactone, one of the major metabolites of EGCg, increased the activity of CD4+ T cells and enhanced the cytotoxic activity of natural killer cells.22) Thus, it is expected catechin metabolites may exhibit additional biological activities. With regard to anticancer effects, 5-(3,4,5-trihydroxyphenyl)−γ-valerolactone has shown growth inhibition of human colon adenocarcinoma cells (HT-29 and HCT-116) and esophageal squamous carcinoma cells (KYSE150).23)

Cervical cancer is one of the most commonly occurring malignant tumors in women. Recently, an increasing number of younger women are contracting this disease and effective treatments are urgently required since the rate of progress is rapid. In general, the main cervical cancer treatments are surgery, radiation therapy and chemotherapy. The cervical cancer treating agents used are bleomycin, paclitaxel, cisplatin, and so on. These treatments can have anticancer effects on the whole body, but they have the risk of side effects such as emesis, hair loss and leukopenia. Tea catechins including (+)-catechin (catechin hydrate) and EGCg have been reported to inhibit proliferation of human cervical cells (SiHa and HeLa cells) and their inhibitory mechanisms were discussed.24–27) However, anti-proliferative activity of catechin metabolites produced by intestinal microbiota against cervical cancer cells is not known.

In this study, we evaluated the effects of catechin metabolites on proliferation of HeLa cells as a model for human cervical cancer.

MATERIALS AND METHODS

Materials The following catechin metabolites were prepared using the methods we reported previously.10–13) 2-(3,5-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3,5,7-triol (EGC-M1), 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M2), 1-(3,5-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M3), 4-hydroxy-5-(3,5-dihydroxyphenyl)valeric acid (EGC-M4), 5-(3,5-dihydroxyphenyl)−γ-valerolactone (EGC-M5), 3-(3,5-dihydroxyphenyl)propionic acid (EGC-M6), 4-hydroxy-5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M7), 5-(3,4,5-trihydroxyphenyl)−γ-valerolactone (EGC-M8), 5-(3,5-dihydroxyphenyl)−γ-valeric acid (EGC-M9), 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EC-M1), 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid (EC-M3), 5-(3,4-dihydroxyphenyl)−γ-valerolactone (EC-M4). All other chemicals were available products of analytical or HPLC grade.

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**Chemical Synthesis of 5-(3,4-Dihydroxyphenyl)levulinic Acid (EC-M7)**

EC-M4 (0.49 mmol, 102.7 mg) and N,N-diisopropylethylamine (1.96 mmol, 510 µL, Tokyo Chemical Ind. Co., Ltd. Tokyo, Japan) were dissolved in 2.0 mL of acetone. To the solution was added chloromethyl methyl ether (1.00 mmol, 158 µL, Kanto Chemical Co., Inc., Tokyo, Japan) and this was agitated for more than 48 h at room temperature. After the adjustment to pH 4.0 with 5% citric acid solution, the reaction solution was added to 3 mL of distilled water and was extracted 3 times with 5 mL of chloroform. The chloroform layer was washed with 5 mL of distilled water and 5 mL of the saturated NaCl solution, and was evaporated to dryness to obtain 5-(3,4-O-dimethoxymethylphenyl)-γ-valerolactone. The compound was dissolved in 1 mL of methanol and 0.1 mL of 3 M KOH solution was added. The reaction mixture was stirred for 1 h at room temperature. To the mixture was added 1 M sodium formate buffer (pH 3.0) to adjust pH to 4.0. After the addition of 3 mL of distilled water, the solution was extracted 3 times with 4 mL of ethyl acetate. Triethylamine (0.1 mL) was added to the ethyl acetate layer and the solution was evaporated to dryness. 4-Hydroxy-5-(3,4-O-dimethoxymethylphenyl)valeric acid trimethylamine salt was obtained. The valeric acid trimethylamine salt was dissolved in 3 mL of dichloromethane and then pyridine (1.83 mmol, 145 µL) and Dess–Martin periodinane (0.92 mmol, 400.0 mg, Sigma-Aldrich Co., LCC, Tokyo, Japan) was added. The reaction mixture was stirred for 4 h at room temperature. To the reaction mixture was added 3 mL of 5% sodium thiosulfate solution and this was agitated for 30 min at room temperature.

After the adjustment to pH 3.0 by adding 5% citric acid solution, the resulting solution was extracted 3 times with 10 mL of dichloromethane. The dichloromethane layer was washed with 10 mL of 5% sodium thiosulfate solution and 10 mL of saturated NaCl solution. The organic layer was dehydrated by sodium sulfate. After filtration, the solution was evaporated to dryness. The residue was dissolved in 10 mL of acetonitrile/distilled water/formic acid (5/95/0.1, v/v/v) and was subjected to a preparative HPLC. The preparative HPLC was performed using CAPCELL PAK MG column (150 mm×20 mm i.d., 5 µm, Shiseido Co., Ltd., Tokyo, Japan) in a Preparative HPLC system PLC791 (GL Sciences Inc., Tokyo, Japan). The column was eluted with mobile phase A (acetonitrile/distilled water/formic acid, 5/95/0.1, v/v/v) and mobile phase B (acetonitrile/distilled water/formic acid, 80/20/0.1, v/v/v) at a flow rate of 9.5 mL/min at 40°C. Initially, the column was eluted with 60% A and 40% B for 3 min, followed by linear increases to 100% B from 3 to 15 min and held at 100% B for 3 min, then eluted with 90% A and 10% B from 18 to 18.5 min, and finally equilibrated with 90% A and 10% B for 3.5 min. Finally, 0.02 mmol (5.5 mg) of 5-(3,4-dihydroxyphenyl)-levulinic acid was obtained and its structure was confirmed by NMR (Ultrasound 400 plus system: 1H, 400 MHz: Bruker) analysis. ^1H-NMR (methanol-d₄): δ: 6.73 (1H, d, J=8.0 Hz), 6.66 (1H, d, J=1.9 Hz), 6.55 (1H, dd, J=8.0, 1.8 Hz), 3.61 (2H, s), 2.76 (2H, t, J=6.3 Hz), 2.50 (2H, t, J=6.5 Hz).

**Chemical Synthesis of 5-(3,4,5-Trihydroxyphenyl)valeric Acid (ECG-M9)**

Five milliliters of dichloromethane was added to 3,4,5-tri-benzoyloxybenzylalcohol (2.23 mmol, 950.5 mg, Tokyo Chemical Ind. Co., Ltd.) and Dess–Martin Periodinane (3.30 mmol, 1.4 g, Sigma-Aldrich Co., LCC) and the mixture was stirred for 3 h at room temperature. The mixture was added to 5 mL of 5% sodium thiosulfate solution and was agitated for 30 min at room temperature. Next, the mixture was extracted with 20 mL of ethyl acetate and the ethyl acetate layer was washed with distilled water, the saturated NaHCO₃ solution, and saturated NaCl solution. After that, the organic layer was dried with sodium sulfate and was filtered. The resulting filtrate was evaporated to dryness and the crude residue containing 3,4,5-tri-benzoyloxybenzaldehyde (1.24 g) was obtained. [3-(Ethoxycarbonyl)propyl]-triphenylphosphonium Bromide (4.38 mmol, 2.0 g, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to 20 mL of anhydrous tetrahydrofuran and the mixture was stirred for 20 min on ice. Tetrahydrofuran containing sodium bis(trimethylsilyl)amide (5.83 mmol, 3.07 mL, Tokyo Chemical Ind. Co., Ltd.) was then added to the mixture and agitated for 1 h on ice. To the resulting solution was added 8 mL of tetrahydrofuran containing 3,4,5-tri-benzoyloxybenzaldehyde (1.24 g synthesized above) and this was stirred for 48 h at room temperature. After adding 30 mL of saturated ammonium chloride, the mixture was extracted with 50 mL of diethyl ether. The organic layer was washed with distilled water, saturated NaHCO₃ solution, and saturated NaCl solution and was dehydrated with sodium sulfate. After removal of sodium sulfate by filtration, the filtrate was evaporated to dryness and the residue was dissolved in 2 mL of methanol–tetrahydrofuran mixture. To the solution was added 0.02 mmol (5.5 mg) of 5-(3,4,5-trihydroxyphenyl)-levulinic acid fraction was collected, concentrated to dryness and finally the purified levulinic acid was obtained (0.06 mmol, 19.1 mg). The compound was dissolved in a mixture of dichloromethane (1.5 mL), distilled water (0.5 mL) and trifluoroacetic acid (1.0 mL) and the solution was stirred for 1 h at room temperature. The mixture was evaporated to dryness and the residue was dissolved in 1 mL of acetonitrile/distilled water/formic acid (5/95/0.1, v/v/v) and then was purified by the same preparative HPLC as above, except that the column was initially eluted with 90% A and 10% B for 3 min, followed by linear
4-pentenoic acid was obtained. The pentanoic acid was dissolved in 8 mL of ethyl acetate and to the solution was added 600.7 mg of Palladium (10 wt% (dry) on carbon powder, wet, Sigma-Aldrich Co., LCC). The mixture was replaced with argon gas and then was stirred for 6 h at room temperature by injecting hydrogen gas. After filtration, the filtrate was evaporated to dryness and the residue was dissolved in 10 mL of acetonitrile/distilled water/formic acid (5/95/0.1, v/v/v) and applied to the preparative HPLC system as described previously. The preparative HPLC system, column, mobile phase, flow rate and absorbance were the same as the synthesis of 5-(3,4-dihydroxyphenyl)levulinic acid. The column was initially eluted with 90% A and 10% B for 3 min, followed by linear increases to 100% B from 3 to 15 min and held at 100% B for 5 min, then eluted with 90% A and 10% B from 20 to 21 min, and finally equilibrated with 90% A and 10% B for 5 min. The 5-(3,4,5-trihydroxyphenyl)valeric acid fraction was collected, concentrated and finally 1.47 mmol (331.7 mg) of the compound was obtained and its structure was confirmed by NMR analysis. 

\(^1\)H-NMR (methanol-\(d_4\)) \(\delta\): 6.23 (2H, s), 2.42 (2H, t, \(J = 6.8\) Hz), 2.31 (2H, t, \(J = 6.7\) Hz), 1.59 (4H, m).

**Chemical Synthesis of 5-(3,4-Dihydroxyphenyl)valeric Acid (EC-M9) and 5-(3-Hydroxyphenyl)valeric Acid (EGC-M11)**

EC-M9 and EGC-M11 were synthesized in a manner similar to 5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M9) except for using 3,4-dibenzyloxybenzaldehyde (2.68 mmol, 851.0 mg, Wako Pure Chemical Industries, Ltd.) for EC-M9 synthesis and m-Benzoxylbenzaldehyde (5.18 mmol, 1.1 g, Wako Pure Chemical Industries, Ltd.) for EGC-M11 synthesis as starting materials. Finally, 0.439 mmol (92.1 mg) of EC-M9 and 2.29 mmol (445.2 mg) of EGC-M11 were obtained and their structures were confirmed by NMR analysis.

EC-M9; 
\(^1\)H-NMR (methanol-\(d_4\)) \(\delta\): 6.67 (1H, d, \(J = 8.0\) Hz), 6.63 (1H, d, \(J = 2.0\) Hz), 6.50 (1H, dd, \(J = 8.0, 2.0\) Hz), 2.49 (2H, t, \(J = 7.0\) Hz), 2.31 (2H, t, \(J = 6.7\) Hz), 1.62 (4H, m). 

EGC-M11; 
\(^1\)H-NMR (methanol-\(d_4\)) \(\delta\): 7.08 (1H, t, \(J = 7.1\) Hz), 6.62 (3H, m), 2.57 (2H, t, \(J = 7.1\) Hz), 2.32 (2H, t, \(J = 7.0\) Hz), 1.64 (4H, m).

**Assay for Inhibitory Activity against HeLa Cell Proliferation**

Catechin metabolites were assayed for cytotoxicity against HeLa cells according to the previous report. Simply stated, HeLa cells (2 \(\times 10^3\) cells/well) were seeded in 96-well microplate and allowed to grow for 24 h. Then, each catechin metabolite in dimethyl sulfoxide (DMSO) was added at a final concentration range of 0.4 to 50 \(\mu\)g/mL and incubated for 72 h at 37°C under an atmosphere of 5% CO\(_2\). Adriamycin in DMSO as a positive control was used at a final concentration range of 6.4 \(\times 10^{-4}\) to 10 \(\mu\)g/mL and DMSO was used as a negative control. After the incubation, each well was added to 50 \(\mu\)L of 1 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in saline and further incubated for 3 h. Then, the plate was centrifuged to remove

Fig. 1. Chemical Structures of Catechin Metabolites and Their Inhibitory Activity on HeLa Cell Proliferation

Inhibitory activity is noted in brackets and is expressed relative to negative control (DMSO) set at 100.
the supernatant, the cells were dissolved in 150 µL of DMSO and the absorbance was measured by a microplate reader at a wavelength of 490 nm.

To determine IC50, HeLa cells were treated with EGC-M9, the most effective inhibitor among catechin metabolites against the proliferation of HeLa cells, and adriamycin was used as a positive control. Final concentration was eight different ranges from 1.28×10⁻³ to 100 µg/mL for the former and 1.28×10⁻⁴ to 10 µg/mL for the later. The IC50 values were calculated from their inhibition curves.

RESULTS AND DISCUSSION

We first screened the inhibitory activities of eleven kinds of metabolites produced from EGC and EGCg by intestinal microbiota on proliferation of human cervical cancer cells (HeLa cells). Figure 1 illustrates the chemical structures of metabolites. Among the metabolites, 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M2), 4-hydroxy-5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M7), and 5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M9) were found to inhibit proliferation of HeLa Cells (Fig. 1) at a final concentration of 50 µg/mL. On the other hand, their analogous metabolites such as 1-(3,5-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M3), 4-hydroxy-5-(3,5-dihydroxyphenyl)valeric acid (EGC-M4), 5-(3,5-dihydroxyphenyl)valeric acid (EGC-M10), and 5-(3-hydroxyphenyl)valeric acid (EGC-M11) did not show any inhibitory activity against HeLa cell proliferation (Fig. 1). These observations suggested that three adjacent hydroxyl groups of the phenyl moiety in the metabolite structures play an important role in the inhibition of cell proliferation. However, 5-(3,4,5-trihydroxyphenyl)-γ-valerolactone (EGC-M8) having the three hydroxyl groups in its phenyl moiety did not exhibit the inhibitory activity, making it an exceptional instance. We have no reasonable explanation for this anomaly but speculate that the valerolactone moiety may be responsible for decreasing affinity for HeLa cells.

In addition, since there are no metabolites possessing two adjacent hydroxyl groups of the phenyl moiety among EGC and EGCG metabolites tested in this study, we examined the inhibitory activity of EC metabolites having two adjacent hydroxyl groups in the phenyl moiety such as 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EC-M1), 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid (EC-M3), 5-(3,4-dihydroxyphenyl)valeric acid (EC-M7) and 5-(3,4-dihydroxyphenyl)valeric acid (EC-M9). As shown in Fig. 1, EC-M1, EC-M3, and EC-M7 did not show inhibitory activity against HeLa cell proliferation whereas EC-M9 inhibited proliferation. Thus, even though EC-M9 has only two adjacent hydroxyl groups in phenyl moiety, it exhibited the inhibitory activity. These results implied that aliphatic side chain, valeric acid in this case, may play a certain role in combination with two adjacent hydroxyl groups in the phenyl moiety. Consequently, it is reasonable that EGC-M9 possessing both valeric acid and three adjacent hydroxyl groups in the phenyl moiety showed strongest inhibitory activity on HeLa cell proliferation.

We examined the IC50 value of EGC-M9 which showed the strongest inhibitory activity, together with that of adriamycin as a positive control. IC50 value of EGC-M9 was calculated to be 5.58 µM (1.26 µg/mL) and was extremely weak as compared with that 0.039 µM (2.1×10⁻² µg/mL) of adriamycin as shown in Fig. 2. However, the value of EGC-M9 was roughly 8.6 times higher than that of EGCG (47.9 µM) which has reported to exhibit the inhibitory activity against HeLa cell proliferation.27) Thus, this compound can contribute to the cancer preventive effect of green tea catechins.

In this study, we newly discovered that EGC-M2, EGC-M7, EGC-M9, and EC-M9 formed from EGC, EGCG, and EC by intestinal microbiota inhibit the proliferation of cervical cancer HeLa cells. The metabolites may be responsible for the cancer preventive activity of green tea as well as EGCG.25–27) Indeed, oral intake of tea catechin has been reported to show a chemopreventive effect against cervical intraepithelial...
neoplasia in human. On the other hand, Lambert et al. reported that EGC-M8 inhibited the cell growth in esophageal squamous carcinoma cells (KYSE150) and human colon adenocarcinoma cells (HT-29), but indicated inhibition of pre-malignant cells by this metabolite could be selective. In our study EGC-M8 showed no inhibitory activity on HeLa cell proliferation, suggesting that selectivity may also be a factor with HeLa cells. Further research is needed not only to evaluate the anticancer effect of catechin metabolites with respect to different cancer cells but also to confirm the effect in vivo experiments.

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Conflict of Interest The authors declare no conflict of interest.

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