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Blood–Brain Barrier Permeability of Green Tea Catechin Metabolites and their Neuritogenic Activity in Human Neuroblastoma SH-SY5Y Cells

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Scope: To understand the mechanism by which green tea lowers the risk of dementia, focus was placed on the metabolites of epigallocatechin gallate (EGCG), the most abundant catechin in green tea. Much of orally ingested EGCG is hydrolyzed to epigallocatechin (EGC) and gallic acid. In rats, EGC is then metabolized mainly to 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (EGC-M5) and its conjugated forms, which are distributed to various tissues. Therefore, we examined the permeability of these metabolites into the blood–brain barrier (BBB) and nerve cell proliferation/differentiation in vitro.
Methods and Results: The permeability of EGC-M5, glucuronide, and the sulfate of EGC-M5, pyrogallol, as well as its glucuronide into the BBB were examined using a BBB model kit. Each brain- and blood-side sample was subjected to liquid chromatography tandem-mass spectrometry analysis. BBB permeability (%, in 0.5 h) was 1.9–3.7%. In human neuroblastoma SH-SY5Y cells, neurite length was significantly prolonged by EGC-M5, and the number of neurites was increased significantly by all metabolites examined.
Conclusion: The permeability of EGC-M5 and its conjugated forms into the BBB suggests that they reached the brain parenchyma. In addition, the ability of EGC-M5 to affect nerve cell proliferation and neuritogenesis suggests that EGC-M5 may promote neurogenesis in the brain.

1. Introduction

Long-term intake of green tea catechins suppresses brain aging.[1–3] Green tea is composed of several catechins. Among them, epigallocatechin gallate (EGCG) is the main molecule, followed by epigallocatechin (EGC). When the suppressive effect of EGCG and EGC on age-related cognitive dysfunction was compared in aged mice, EGCG was as effective as the catechin mixture, but EGC had little effect.[4] The bioavailability of EGCG is estimated to be 0.1∼0.26% after oral ingestion in rats and humans.[5–7] To understand the mechanism of action of EGCG in the brain, its permeability of the blood–brain barrier (BBB) was examined using a BBB model kit.[4] That study indicated that the in vitro permeability (%, in 0.5 h) of EGCG and EGC was 2.8 and 3.4%, respectively.[4] In addition, the growth of human neuroblastoma, SH-SY5Y cells, was significantly increased by 0.05 μM EGCG, but this effect was reduced at higher concentrations of EGCG. The effect of EGC was lower than that of EGCG at 0.05 μM. These results suggested that a very low concentration of EGCG, when incorporated into brain parenchyma via the BBB, enhanced nerve cell proliferation and differentiation.

Epidemiological studies demonstrated that daily ingestion of one or two cups of green tea significantly reduces the risk of dementia.[8,9] If our BBB permeability data were to be applied to humans, the concentration of EGCG is speculated be approximately 0.01 μM in their brain after ingesting one or two cups of green tea. As EGCG disappears within several hours from plasma,[6,7,10,11] the level of EGCG may be insufficient to prevent dementia.

On the other hand, much of orally ingested EGCG is hydrolyzed by intestinal microbiota to EGC and gallic acid (GA) while most EGC is degraded in the large intestine to 5–(3′,5′-dihydroxyphenyl)-γ-valerolactone (EGC-M5) by intestinal bacteria in rats.[6] A large part of EGC-M5 that is formed is absorbed and glucuronidated in the intestinal mucosa and/or liver to form the glucuronide of EGC-M5 (EGC-M5-GlcUA), which is distributed to various tissues via blood circulation and is

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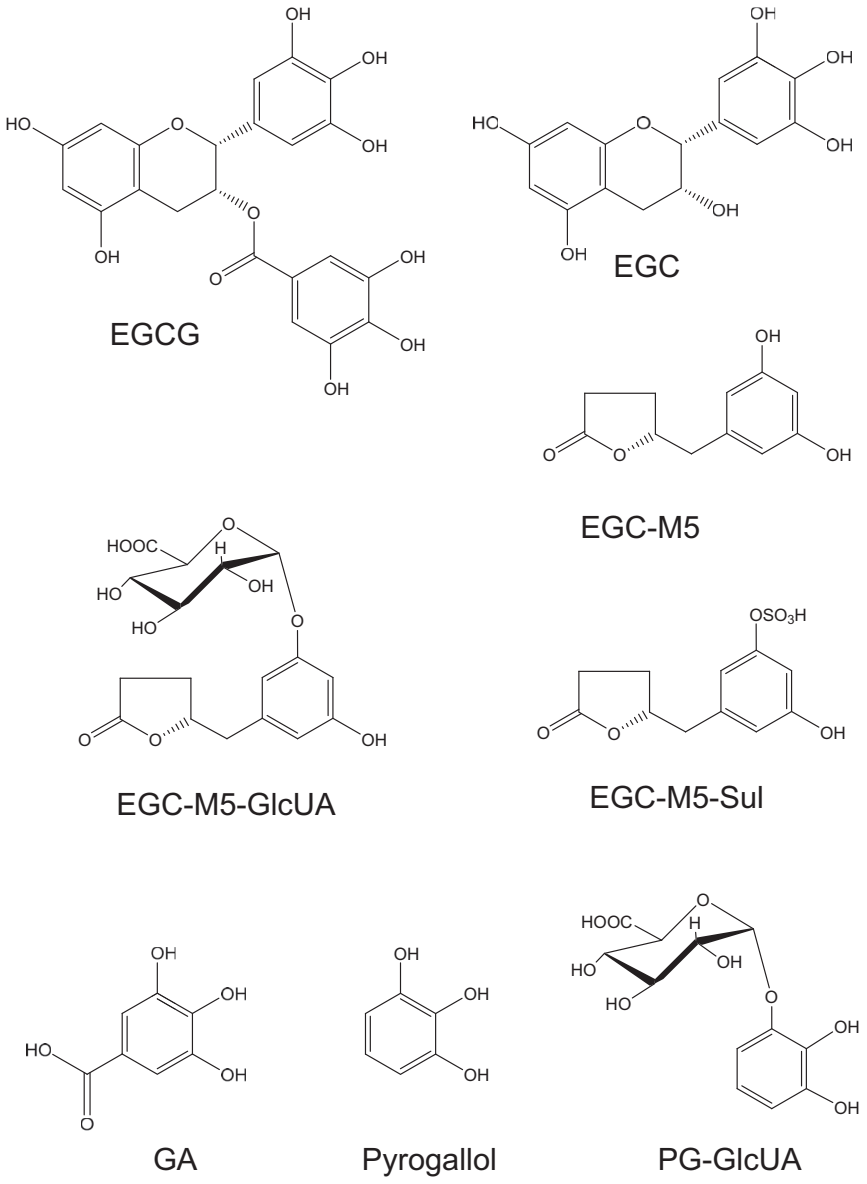

Figure 1. Structures of EGCG metabolites used in this experiment.

finally excreted in urine.[6] A human urinary metabolite profile of tea polyphenols also demonstrated that the conjugates of the ring-fission metabolites of catechins, such as EGC-M5, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, were the major urinary metabolites of tea polyphenols.[10] As approximately two-thirds of ingested tea polyphenols pass from the small to the large intestine in humans,[11] some of these large intestine-derived ring-fission metabolites of EGCG may play an important role in vivo. In addition, pyrogallol (PG) was detected as a degradation product of GA using an ex vivo model.[12] GA is suggested to be absorbed from the small intestine while PG is absorbed from the large intestine.[13] If EGCG metabolites similarly promote the differentiation of nerve cells, this may partially explain why the daily intake of one or two cups of green tea effectively prevents dementia.

In order to better understand the mechanism of action of EGCG in the brain, the in vitro BBB permeability of EGCG metabolites, such as EGC-M5, PG, and their conjugated forms, as well as their effect on nerve cell proliferation and differentiation, were examined in this study. Based on these data, we considered the role of EGCG metabolites in the brain.

2. Experimental Section

2.1. Preparation of EGC-M5, Glucuronide, and Sulfate of EGC-M5 (EGC-M5-GlcUA and EGC-M5-Sul), and Glucuronide of PG (PG-GlcUA)

EGC-M5 (**Figure 1**), a biological metabolite of EGCG, was prepared according to a previously reported method.[14] In brief, EGC

(Figure 1, Sigma–Aldrich, Tokyo, Japan) was incubated with rat intestinal flora for 96 h under anaerobic conditions. EGC-M5 produced in the incubation medium was purified using preparative HPLC [150 mm × 20 mm id, 5 μm, Capcellpak C18 MG column (Shiseido Co. Ltd., Tokyo, Japan)] in a JASCO HPLC 800 series system (JASCO, Tokyo, Japan).

EGC-M5-GlcUA and PG-GlcUA (Figure 1) were prepared from the urine of rats (Wistar, male, 36 weeks, Japan SLC Inc., Shizuoka) that were orally administered EGC-M5 or PG (Figure 1), respectively. All experimental procedures were in accordance with the guidelines for animal experiments of Food Research Laboratories, Mitsui Norin Co. Ltd. (Permission number 2014-012). EGC-M5-Sul (Figure 1) was prepared as follows: EGC-M5 was stirred with dried pyridine and sodium sulfate for 10 min at room temperature. This mixture was then stirred with pyridine sulfur trioxide for 1 h at room temperature. Details of the purification and identification of these compounds may be found in the Supporting Information.

2.2. Measurement of BBB Permeability

A BBB kit (RBT-24, PharmaCo-Cell, Nagasaki, Japan), consisting of cocultures of endothelial cells, pericytes, and astrocytes, was used to determine BBB permeability rates and coefficients of EGC-M5, its conjugates, PG, and its glucuronide. According to this method's protocol, the BBB kit was first activated. After transepithelial electrical resistance reached >150 $\Omega \times cm^2$ (Endohm^{TM}, World Precision Instruments Inc., USA), the kit was used to measure permeability.

EGC-M5 and other samples were dissolved in DMSO and a 1 μL aliquot was added to the blood side of the BBB kit at a final concentration of 30 μM, as described previously.[4] The plate was incubated for 30 min with gentle stirring, then 190 μL of blood-side medium and 380 μL of brain-side medium were added to 10 and 20 μL of McIlvain buffer (pH 3.0), respectively in a tube, and frozen immediately. To reduce variability among plates, the same experiment was carried out three times. The samples were stored at −80 °C until analysis. The concentrations of EGC-M5 and other samples were measured using liquid chromatography tandem-mass spectrometry (LC–MS/MS). Based on the concentrations of blood- and brain-side samples, permeability was calculated according to the method described in the kit's protocol.

2.3. Measurement of Catechins Using LC–MS/MS

Each brain- and blood-side sample (90 μL) was mixed with 10 μL of McIlvain buffer (pH 3.0) as described previously.[4] In brief, after the supernatants (12 000 × *g*, 5 min) were filtered with a 0.45 μm hydrophilic PTFE filter (DISMIC-13HP, ADVANTEC Toyo, Tokyo, Japan), they were subjected to LC–MS/MS analysis.[4]

2.4. Cell Growth Assay in Vitro

Human SH-SY5Y neuroblastoma cells (ACTT, CRL-2266) were plated in a 100-mm flask and cultured in D-MEM/Ham's F-12 with L-glutamine, phenol red, HEPES, and sodium pyruvate (Wako Pure Chemical Industries Ltd., Osaka, Japan), containing 10% fetal bovine serum (Mediatech Inc., Tokyo, Japan) and a mixture of 1% penicillin–streptomycin (Nacalai Tesque Inc., Kyoto, Japan). The cell culture was incubated at 37 °C under 5% CO_2 for 48 h, as described previously.[4] The cells were plated as 1×10^5 cells/mL in a 24-well plate (500 μL of cell suspension/well). EGC-M5 and other samples dissolved in 0.01% DMSO were added to the culture medium to make a final concentration of 0.01–1.0 μM in triplicate for each concentration. Plates were incubated for 48 h. Cells were counted with a TC10^{TM} Automated Cell Counter (Bio-Rad, CA, USA). The experiment was carried out twice for each sample.

2.5. Quantitation of Neurite Outgrowth

SH-SY5Y cells were plated as 2.5×10^4 cells/mL in a 24-well plate (500 μL of cell suspension/well). EGC-M5 and other samples dissolved in 0.01% DMSO were added to the culture medium to make a final concentration of 0.05 μM, and cultured for 72 h. Cells were visualized by using a phase-contrast inverted microscope (IX71; Olympus, Tokyo, Japan) with a LUCPIanFLN 20×/0.45 objective lens (Olympus) and a DP70 digital microscope camera (Olympus). Neurite length was measured by ImageJ software (Ver. 1.50i), as described previously.[4] Assays were performed in triplicate and at least three photos from each culture were taken.

2.6. Statistical Analyses

Data are expressed as the mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. ANOVA was assessed using a statistical analysis program (StatPlus, AnalystSoft Inc., online version). Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. BBB Permeability of EGC-M5 and Other Metabolites

The concentrations of EGCG metabolites in blood and brain sides were measured by LC–MS/MS (**Table 1**). The recovery of each was almost 100%, indicating that no degradation occurred during incubation for 0.5 h. Based on these concentrations, permeability was calculated (**Table 2**). EGC-M5 exhibited a slightly higher permeability coefficient and permeability (%, in 0.5 h) than EGC and EGCG. The permeability of EGC-M5 conjugates, such as EGC-M5-GlcUA and EGC-M5-Sul, was lower than EGC-M5. The permeability of PG and PG-GlcUA was significantly lower than that of GA. These results suggest that EGC-M5, its conjugates, and PG were transferred into the brain parenchyma.

Table 1. Quantitative analysis of EGCG metabolites in BBB permeability test.

Sample (30 μM)	Blood side (μM)	Brain side (μM)	Recovery (%)
EGC-M5	27.3 ± 0.27	0.25 ± 0.004	103.7 ± 0.06
EGC-M5-GlcUA	28.1 ± 0.22	0.17 ± 0.0005	102.6 ± 0.01
EGC-M5-Sul	27.9 ± 0.01	0.20 ± 0.02	103.0 ± 0.30
PG	24.9 ± 0.14	0.19 ± 0.02	103.1 ± 0.42
PG-GlcUA	28.2 ± 0.64	0.09 ± 0.04	101.3 ± 0.67

Data are expressed as the mean ± SEM ($n = 3$).

Table 2. BBB permeability of EGCG metabolites.

Sample	Permeability coefficient (10^{-6} cm s^{-1})	BBB permeability (%) (30 min)
EGC-M5	12.45 ± 0.45	3.70 ± 0.13
EGC-M5-GlcUA	8.68 ± 0.01	2.58 ± 0.00
EGC-M5-Sul	10.11 ± 0.76	3.00 ± 0.23
PG	9.55 ± 0.92	2.84 ± 0.27
PG-GlcUA	4.30 ± 0.98	1.91 ± 0.29
[a] EGCG	9.31 ± 0.32	2.77 ± 0.10
[a] EGC	11.56 ± 1.05	3.43 ± 0.31
[a] GA	21.97 ± 1.92	6.52 ± 0.57

[a] These data are from Ref. [4] Data are expressed as the mean ± SEM ($n = 3$).

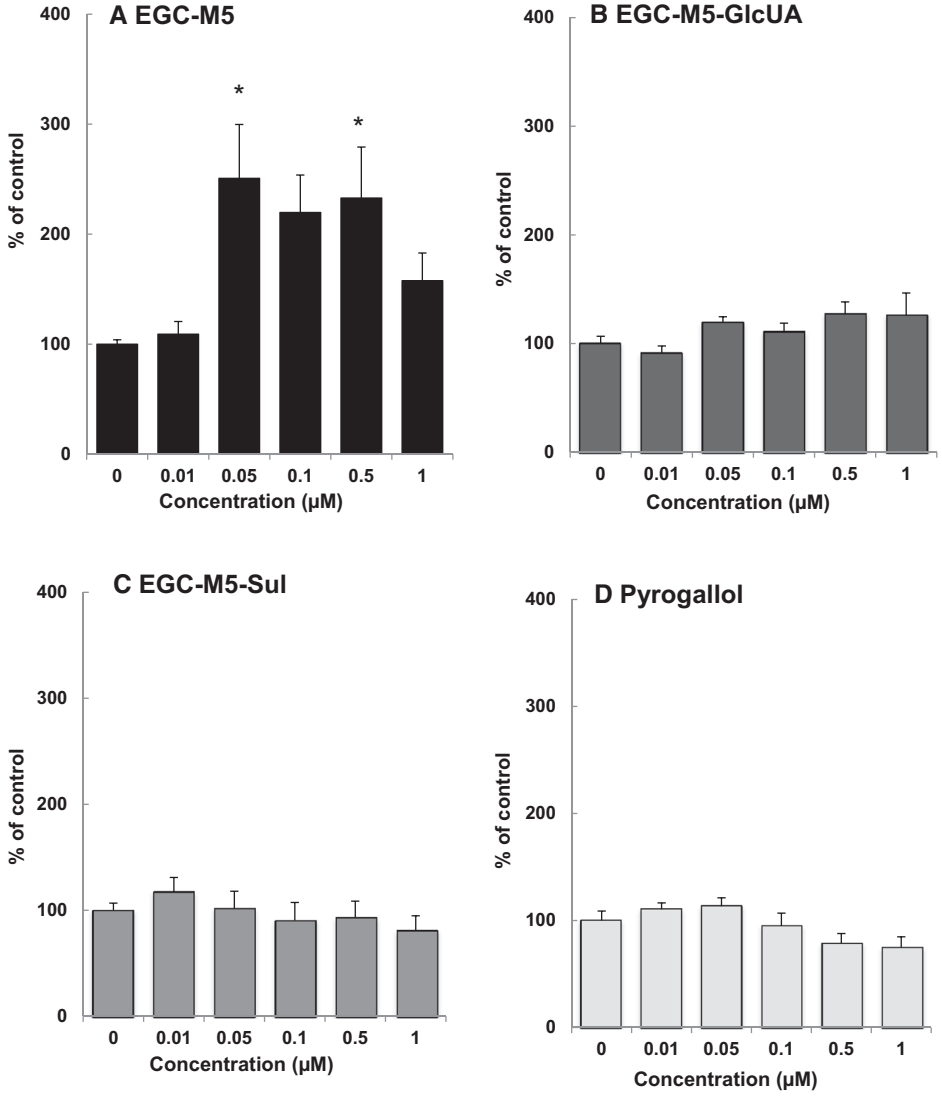

Figure 2. Effect of EGC-M5, EGC-M5-GlcUA, EGC-M5-Sul, and PG on the growth of human SH-SY5Y neuroblastoma cells. A cell suspension (5×10^4 cells/well) was placed in a 24-well plate. EGC-M5 and other samples dissolved in 0.01% DMSO were added to the culture medium to make a final concentration of 0.01–1.0 μM, and cultured for 48 h at 37 °C. The number of cells treated with EGC-M5 (a), EGC-M5-GlcUA (b), EGC-M5-Sul (c), and PG (d) are shown. Each value represents the mean ± SEM ($n = 6$). Asterisks represent significant differences relative to the control (*$p < 0.05$, Bonferroni's *t*-test).

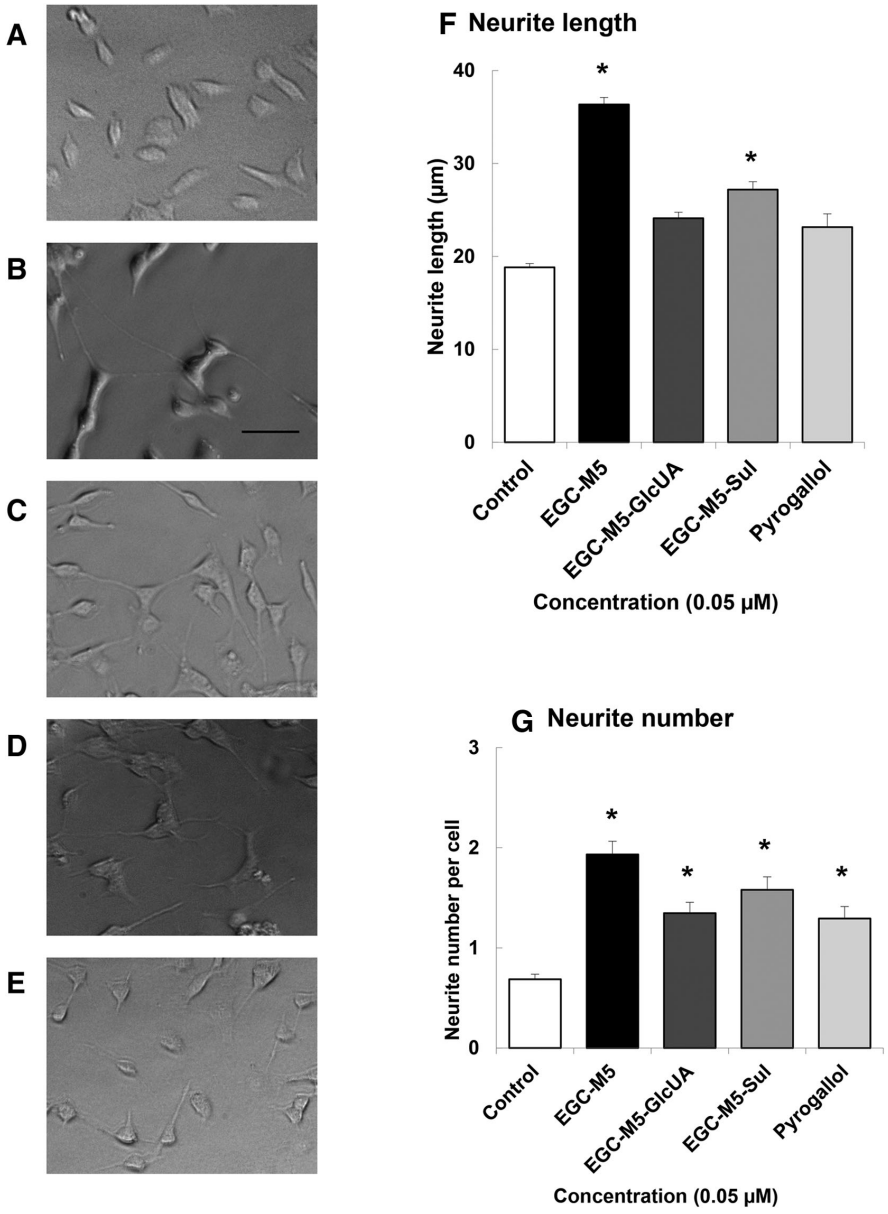

Figure 3. Effect of EGC-M5, EGC-M5-GlcUA, EGC-M5-Sul, and PG on neurite outgrowth of human SH-SY5Y neuroblastoma cells. A cell suspension (2.5×10^4 cells/well) was plated in a 24-well plate. EGC-M5 and other samples dissolved in 0.01% DMSO were added to the culture medium to make a final concentration of 0.05 μM, and cultured for 72 h at 37 °C. Photos of control cells (a), and cells treated with EGC-M5 (b), EGC-M5-GlcUA (c), EGC-M5-Sul (d), PG (e), Neurite length (f), and neurite number (g) of cells treated with EGCG metabolites. Scale bar is 50 μm. Each value represents the mean ± SEM (neurite length, $n = 103–107$; neurite number, $n = 67–109$). Asterisks represent significant differences relative to the control (*$p < 0.05$, Bonferroni's *t*-test).

3.2. Effect of EGC-M5, its Conjugates, and PG on Nerve Cell Growth and Neurite Outgrowth in SH-SY5Y Cells

EGC-M5 enhanced SH-SY5Y cell number at 0.05 μM (**Figure 2**). The effect was lower in the presence of ≥1.0 μM EGC-M5, indicating that there is an optimal concentration of EGC-M5 for cell proliferation. EGC-M5-GlcUA, EGC-M5-Sul, and PG had no effect on cell growth. PG-GlcUA was not examined because of its very poor BBB permeability.

Next, the neuritogenic ability of EGC-M5 and other metabolites on SH-SY5Y cells was compared at 0.05 μM. The length and number of neurites in cells treated with EGC-M5 and EGC-M5-Sul increased significantly compared to control cells (**Figure 3**). In SH-SY5Y cells treated with the glucuronide of EGC-M5 and PG,

the number of neurites was significantly higher than the control, although neurite length did not increase significantly.

4. Discussion

To understand the mechanism of action of EGCG in the brain, BBB permeability of EGCG metabolites, and nerve cell proliferation/differentiation in vitro were examined. LC–MS/MS analysis indicated that these EGCG metabolites were stable for 0.5 h, suggesting that they may stably circulate in blood and reach the brain parenchyma through the BBB. EGC-M5, with smaller molecular size than EGCG, showed slightly higher permeability than EGCG and EGC. On the other hand, the permeability of the conjugated forms of EGC-M5 and PG that increased hydrophilicity were slightly lower than their corresponding aglycons. The permeability of PG was significantly lower than that of GA, despite the higher hydrophobicity of PG than GA, because GA is incorporated into cells through an organic anion transporter.[15] As the main purpose of this study was to compare the permeability of catechin metabolites, a time-dependent kinetics study was not conducted. A further time-dependent analysis of the metabolites in plasma and brain will be needed to provide more reliable information.

The BBB permeability of EGC-M5, its conjugates, and PG suggested their transfer into the brain parenchyma. Among them, significant proliferation and neuritogenic activities were observed in cells treated with EGC-M5. EGC-M5 may have the same effect as EGCG in the brain.[4]

A portion of EGCG is absorbed from the small intestine without the loss of the galloyl moiety and appears in the circulation system, showing a peak in concentration 1.6–2.3 h after intake, but only being present in trace amounts after 8 h.[6,7,10,11] However, >8 h after ingestion, the remaining EGCG that reaches the large intestine is catabolized to ring-fission metabolites, such as EGC-M5.[14] Based on previous data,[6] the concentration of EGC-M5 (free/conjugates) in plasma is assumed to be approximately 0.2%. When humans drink green tea, both intact EGCG and metabolized EGCG may promote the differentiation of neurons. Although the first action of intact EGCG may be important, after EGCG disappears, metabolized EGCG, EGC-M5, and its conjugates may increase neurite outgrowth. Alternatively, EGCG metabolites that enter the brain may have a role in lowering oxidative damage. Indeed, the levels of lipid peroxidation in the brain were significantly lower in mice that ingested EGCG or EGC than in control mouse brains.[4] In addition, EGCG metabolites degraded by rat intestinal flora have been confirmed to have antioxidative activity.[16]

Many studies on the biological activity of intestinal metabolites are needed to explain the health benefit effects of tea catechins. Recently, it has been reported that two metabolites of EGCG, EGC-M5 and 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, have hypotensive effects in vivo.[17] EGC-M5 increased the activity of $CD4^+$ T cells and enhanced the cytotoxic activity of NK cells in vivo, but the effect of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone on immune activity was low.[18] The production of nitric oxide was inhibited by 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone.[19] Catechin metabolites, such as 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol, 4-hydroxy-5-(3,4,5-trihydroxyphenyl) valeric acid, and 5-(3,4,5-trihydroxyphenyl) valeric acid showed inhibitory activity on HeLa cell proliferation, unlike the control.[20] In addition to these studies, how differences in intestinal microbiota affect metabolism needs to be investigated.

In conclusion, EGC-M5, the main ring-fission metabolite of EGCG, showed slightly higher BBB permeability than EGCG and had neuritogenic activity in SH-SY5Y cells at 0.05 μM. Ring-fission metabolites of EGCG in the large intestine, such as EGC-M5, may have neurogenetic activity in the brain as similar as EGCG.

Abbreviations

BBB, blood–brain barrier; EGC, epigallocatechin; EGCG, epigallocatechin gallate; EGC-M5, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; EGC-M5-GlcUA, glucuronide of EGC-M5; EGC-M5-Sul, sulfate of EGC-M5; GA, gallic acid; LC–MS/MS, liquid chromatography tandem-mass spectrometry; PG, pyrogallol; PG-GlcUA, glucuronide of PG; SH-SY5Y, a human neuroblastoma cell

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgments

K.U. F.N. and Y.N. supervised the study. M.P., A.N., K.I., A.H., A.T. and F.N. carried out of BBB permeability measurements. M.P. and A.M. carried out the experiments using cultured cells. A.Y., A.T. and F.N. prepared EGCG metabolites. K.U., A.H., A.T. and F.N wrote the manuscript. This research study was supported by a JSPS KAKENHI Grant Number JP15K00828, Mishima Kaiun Memorial Foundation and a grant for specifically promoted research of the University of Shizuoka.

Conflict of Interest

The authors declare no conflicts of interest.

Keywords

5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, blood–brain barrier, EGCG, green tea catechin, nerve cell proliferation/differentiation

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