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Relationship between the Biological Activities of Methylated Derivatives of (−)-Epigallocatechin-3-*O*-gallate (EGCG) and Their Cell Surface Binding Activities

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It was previously reported that (−)-epigallocatechin-3-*O*-gallate (EGCG) suppresses the expression of the high-affinity IgE receptor FcεRI in human basophilic cells and that this suppressive effect is associated with EGCG binding to the cell surface. This study examined the effects of five methylated derivatives of EGCG, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate (EGCG 3″Me), (−)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (EGCG 4″Me), (−)-4′-*O*-methyl-epigallocatechin-3-*O*-gallate (EGCG 4′Me), (−)-epigallocatechin-3-*O*-(3,4-*O*-methyl)gallate (EGCG 3″4″diMe), and (−)-4′-*O*-methyl-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (EGCG 4′4″diMe) on FcεRI expression and ERK1/2 phosphorylation, and each of their cell surface binding activities was measured. Of these five methylated derivatives, three that are methylated at the 3″- and/or 4″-position, EGCG 3″Me, EGCG 4″Me, and EGCG 3″4″diMe, suppressed FcεRI expression and ERK1/2 phosphorylation, although the suppressive effects were lower than that of EGCG. EGCG 4′Me and EGCG 4′4″diMe, both of which are methylated at the 4′-position, did not demonstrate a suppressive effect. Furthermore, it was found that EGCG 3″Me, EGCG 4″Me, EGCG 3″4″diMe, and EGCG 4′Me, which are methylated at the 3″- and/or 4″-positions or the 4′-position, could bind to the cell surface even though their binding activities were lower than that of EGCG. Only EGCG 4′4″diMe, which is methylated at both the 4′- and 4″-positions, could not bind. These results suggest that the trihydroxyl structure of the B ring is essential for EGCG to exert the suppressive effects and that the hydroxyl groups on both the 4′-position in the B ring and the 4″-position in the gallate are crucial for the cell surface binding activity of EGCG.

KEYWORDS: Methylated derivatives of EGCG; FcεRI; ERK1/2; cell surface binding activity

INTRODUCTION

Tea (*Camellia sinensis* L.) has been consumed worldwide since ancient times. The three most common types, nonfermented green tea, semifermented oolong tea, and fermented black tea, are derived from leaves of the same tea plant with the characteristics of each determined by the manufacturing processes. It has been reported that tea has various bioregulatory activities, such as antioxidative (*1*−*3*), anticancer (*4*−*7*), and antiallergic (*8*−*14*) activities. The principal group of compounds responsible for these activities was shown to be the catechins, a group of polyphenolic compounds. We previously reported that (−)-epigallocatechin-3-*O*-gallate (EGCG), the major polyphenol in green tea, suppressed the expression of the high-affinity IgE receptor FcεRI by inhibiting the phosphorylation of the extracellular signal-regulated kinase1/2 (ERK1/2) (*8*, *15*). Furthermore, our previous paper indicated that EGCG exerts the suppressive effects by binding to the cell surface in human basophilic cell lines (*15*).

Recent research has shown that the *O*-methylated derivatives of EGCG, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate (EGCG 3″Me) and (−)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (EGCG 4″Me), which are isolated from Tong ting oolong tea, which is a Taiwanese oolong tea product, and Benifuki cultivar, which is one of the cultivars used for Japanese black tea, inhibited type I and type IV allergies (*16*, *17*). It has been demonstrated that these two methylated derivatives and (−)-4′-*O*-methyl-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (EGCG 4′4″diMe), which was detected in the blood and urine samples of humans, mice, and rats after oral administration of green tea (*18*, *19*),

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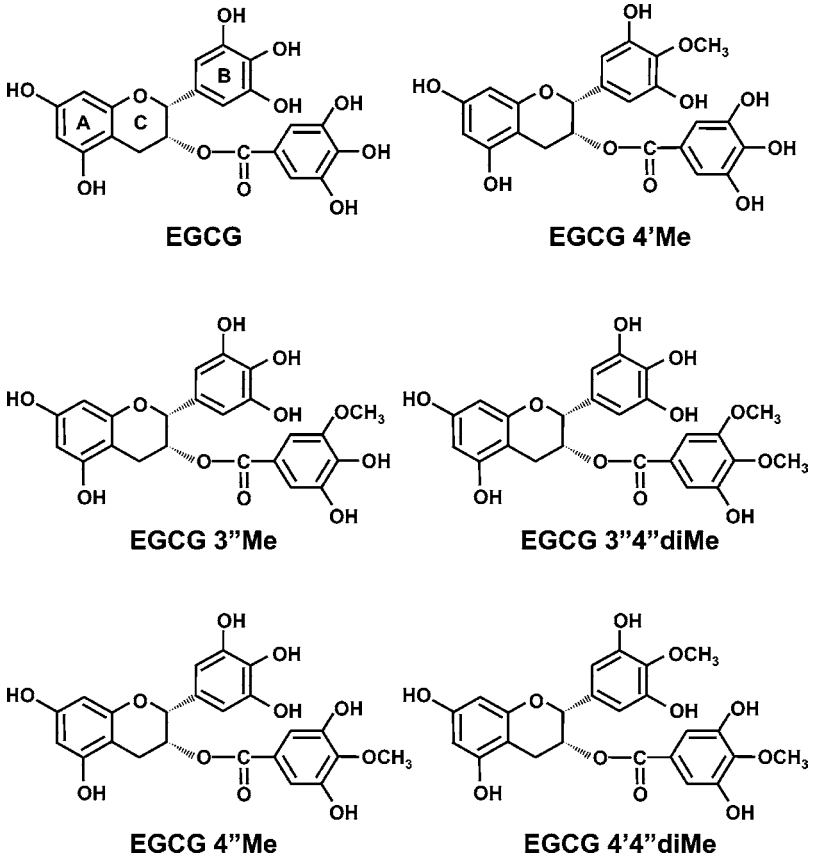

Figure 1. Chemical structures of EGCG and the methylated derivatives of EGCG.

have an inhibitory effect on nitric oxide generation and inducible nitric oxide synthase expression (*20*). However, little is known about the relationship between the biological activities of the methylated derivatives of EGCG and their cell surface binding activities. Recently, we have reported that the inhibitory effect of EGCG on tumor cell proliferation is exerted by EGCG binding to the cell surface via the 67 kDa laminin receptor (67LR) (*21*). This suggests that the biological activities of EGCG are mediated by the target molecule 67LR.

In this study to elucidate the structure−function relationship of EGCG, we examined the effects of the methylation of EGCG on cell surface binding and measured the corresponding biological response using the five methylated derivatives of EGCG.

MATERIALS AND METHODS

Reagents. EGCG, EGCG 3″Me, and EGCG 4″Me were isolated from green tea leaves or Tong ting oolong tea according to the method previously reported (*16, 22*). EGCG 3″4″diMe and EGCG 4′4″diMe were prepared as follows. EGCG was dissolved in dimethylformamide and methylated with methyl iodide and lithium carbonate under stirring for 7 days at room temperature. The reaction mixture was acidified with diluted hydrochloride and chromatographed on a Diaion HP-20 column after dilution with water. The column was washed with water, and the adsorbed material was eluted with methanol to give a pale brown residue. This residue was chromatographed on a silica gel column to give three fractions (A−C) eluting with chloroform/methanol (92:8). Half of fraction A was subjected to preparative HPLC [column, TSKgel ODS-80TS; solvent, CH_3CN/H_2O (80:20−72:28) linear gradient (16 h); UV, 280 nm] to give the colorless and amorphous EGCG 3″4″diMe and EGCG 4′4″diMe. EGCG 4′Me was prepared as follows. EGCG was dissolved in methanol and methylated with diazomethane ether solution at −20 °C for 1 h. The reaction mixture was concentrated and subjected to preparative HPLC [column, TSKgel ODS-80TS; solvent, H_2O/CH_3CN (85:15); UV, 280 nm] to give the colorless and amorphous EGCG 4′Me. The purity (≥95%) of EGCG and all of the methylated derivatives was confirmed by ^{1}H NMR. Mouse anti-human FcεRIα chain monoclonal antibody CRA-1 was obtained from Kyokuto Seiyaku (Tokyo, Japan). Mouse IgG2b antibody, which was used as a negative control, was purchased from Dako. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody was purchased from Protos Immunoresearch (Burlingame, CA). Mouse anti-phosphorylated ERK1/2 antibody and rabbit anti-ERK1/2 antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody and HRP-conjugated anti-rabbit antibody were obtained from Zymed Laboratories, Inc. (San Francisco, CA) and ICN Pharmaceuticals, Inc. (Aurora, OH), respectively.

Cell Culture. KU812 is a human basophilic cell line, which has characteristics of basophilic leukocytes (FcεRI expression, basophilic granules, histamine production). The cells were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan) and were maintained in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY), 100 units/mL penicillin G, 100 mg/mL streptomycin, and 10 mM HEPES buffer. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Flow Cytometric Analysis of Cell Surface FcεRI Expression. KU812 cells (1×10^6 cells/mL) were cultured in serum-free RPMI 1640 medium with or without 25 μM of one of the methylated derivatives of EGCG for 24 h. To assess the cell surface expression of FcεRI, flow cytometry was performed as described in our previous paper (*23*).

Immunoblot Analysis for ERK1/2 Phosphorylation. KU812 cells (1×10^6 cells/mL) were cultured with 25 μM of one of the methylated derivatives of EGCG for 3 h under serum-free conditions. Cells were rinsed once with PBS and lysed in 1% Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL aprotinin, and 1 mM pervanadate). Insoluble material was removed by centrifugation at 12000*g* for 20 min at 4 °C. Prior to analysis, total protein in the cell lysates was measured using a colorimetric BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL) against bovine serum albumin standards. To detect the ERK1/2 phosphorylation, immunoblot analysis was performed as described in our previous paper

(*23*). The density of each band was quantified using a computer program obtained from the U.S. NIH.

Binding Analysis by SPR. Analysis of the interaction between one of the methylated derivatives of EGCG and KU812 cells was performed using the surface plasmon resonance (SPR) biosensor SPR670 (Moritex Corp., Tokyo, Japan). Sensor chips with 50 nm thick gold films were irradiated with ultraviolet. After activation of the sensor chip with ethyl dimethylaminopropyl carbodiimide hydrochloride and *N*-hydroxysuccinimide, KU812 cells (6×10^3 cells) were immobilized on the sensor chip. This chip was equilibrated in PBS at a flow rate of 15 μL/min. EGCG or one of the methylated derivatives (60 μL) diluted in PBS was injected at a flow rate of 15 μL/min at 25 °C. The value of resonance units (RU) in **Figure 4** corresponds with the binding strength.

RESULTS

Effect of the Methylated Derivatives of EGCG on the Cell Surface Expression of FcεRI. Our previous papers demonstrated that EGCG and EGCG 3″Me suppressed the cell surface FcεRI expression by down-regulating the expression of mRNA in KU812 cells (*8*, *9*). We first investigated the suppressive effect of the other methylated EGCG derivatives on FcεRI expression. **Figure 1** shows the structures of the five methylated derivatives of EGCG examined in this study. EGCG has flavan-3-ol structure with A and B rings and a galloyl group. KU812 cells were cultured for 24 h with each of these methylated derivatives. The results of the flow cytometric analysis for cell surface FcεRI expression have been expressed as a relative inhibitory index as shown in **Figure 2**. EGCG 3″Me, EGCG 4″Me, and EGCG 3″4″diMe demonstrated the ability to suppress cell surface expression of FcεRI, but the suppressive effect for each derivative was lower than that of EGCG itself. Interestingly, EGCG 4′Me and EGCG 4′4″diMe, both of which have a methyl ether group in the 4′-position of the B ring, could not suppress FcεRI expression. These results suggest that the 4′-position of the B ring is critical for EGCG's ability to suppress the cell surface expression of FcεRI.

Effect of the Methylated Derivatives of EGCG on the Phosphorylation of ERK1/2. In our previous work, EGCG was shown to decrease the level of ERK1/2 phosphorylation and that the reduction of ERK1/2 phosphorylation may be involved in the down-regulation of FcεRI expression (*15*). Then, we investigated the effect of each methylated derivative of EGCG on the ERK1/2 phosphorylation in KU812 cells. Cells were treated with one of the methylated derivatives for 3 h, and the levels of phophorylated ERK1/2 were assessed by immunoblot analysis. As shown in **Figure 3**, EGCG 3″Me, EGCG 4″Me, and EGCG 3″4″diMe, which all suppressed the cell surface expression of FcεRI, also showed reduced phosphorylated ERK1/2 levels, and the strength of inhibition of ERK1/2 phosphorylation for each derivatives was lower than that for EGCG itself. Again, interestingly, EGCG 4′Me and EGCG 4′4″diMe, which have above shown not to have the ability to suppress FcεRI expression, also demonstrated the inability to reduce the level of ERK1/2 phosphorylation. These results suggest that the reduction of ERK1/2 phosphorylation is involved in the down-regulation of FcεRI expression and that the methylation attenuates the inhibitory effect of EGCG on ERK1/2 phosphorylation. Especially, the methylation of the 4′-position of the B ring is shown to be a crucial site for abrogating the inhibitory effects of EGCG via ERK1/2 phosphorylation.

Cell Surface Binding Activities of the Methylated Derivatives of EGCG. We previously demonstrated that EGCG binds to the cell surface of KU812 cells by the SPR analysis (*15*). To examine whether the methylated derivatives of EGCG actually bind to the cell surface, we again performed SPR analysis. After the immobilization of KU812 cells on a sensor chip, EGCG or one of the methylated derivatives was tested. As shown in **Figure 4**, EGCG 3″Me, EGCG 4″Me, EGCG 4′Me, and EGCG 3″4″diMe were able to bind to the cell surface, but the strength of binding for each derivative was lower than that of EGCG itself. On the other hand, EGCG 4′4″diMe was shown not to be able to bind to the cell surface. These results suggest that the cell surface binding activity of EGCG is affected by the methylation and the methylated position.

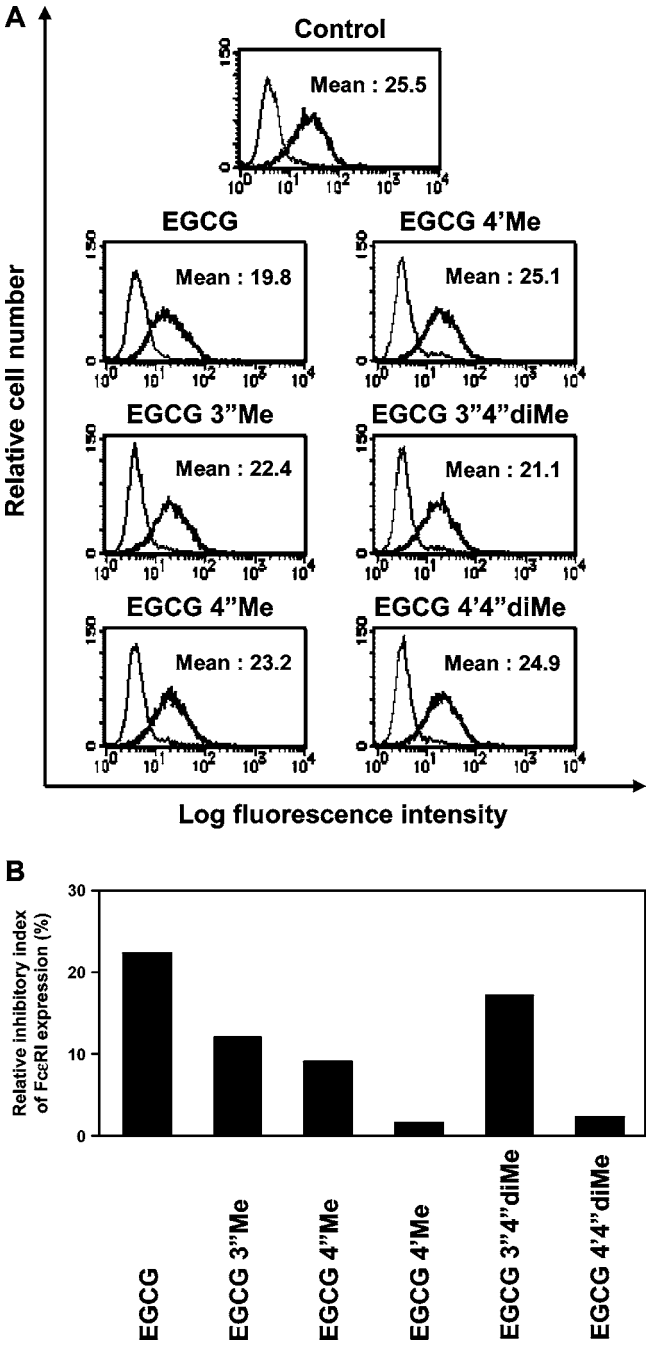

Figure 2. Effect of methylated derivatives of EGCG on cell surface expression of FcεRI. KU812 cells were cultured with each methylated derivative (25 μM) for 24 h. Cells were examined by immunolabeling using the anti-FcεRIα chain antibody followed by staining with the FITC-conjugated goat anti-mouse IgG antibody. Mouse IgG2b antibody was used as the isotype-matched negative control. Data acquisition was performed on a FACSCalibur flow cytometer (**A**). Inhibitory index of FcεRI expression expressed as a percentage of reduction compared to the mean fluorescence intensity of control (**B**).

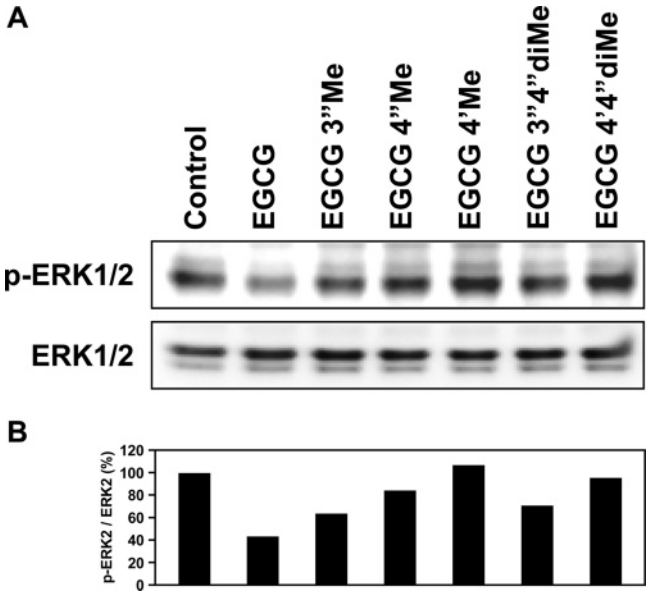

Figure 3. Effects of one of each of the methylated derivatives of EGCG on ERK1/2 phosphorylation in KU812 cells. KU812 cells were cultured in the presence of each methylated derivative (25 μM) for 3 h. The cell lysates were prepared and subjected to 10% SDS-PAGE. Phosphorylated ERK1/2 was detected by immunoblotting using the anti-phospho-ERK1/2 or anti-ERK1/2 antibody (**A**). Relative phosphorylation levels of ERK2 expressed as a percentage compared to the intensity of control (**B**).

DISCUSSION

We examined the effects of five methylated derivatives of EGCG on FcεRI expression and ERK1/2 phosphorylation. EGCG 3″Me, EGCG 4″Me, and EGCG 3″4″diMe were shown to be able to suppress the cell surface expression of FcεRI, although the strength of suppression for these three methylated derivatives was lower than that for EGCG itself. On the other hand, EGCG 4′Me and EGCG 4′4″diMe had no effect on FcεRI expression. Furthermore, **Figure 3** shows that EGCG 3″Me, EGCG 4″Me, and EGCG 3″4″diMe all showed the ability to decrease ERK1/2 phosphorylation, but both EGCG 4′Me and EGCG 4′4″diMe could not. The effects of these five methylated derivatives on the cell surface expression of FcεRI correlated very well with their effects on the ERK1/2 phosphorylation. In our previous study, we demonstrated that (−)-epicatechin-3-*O*-gallate (ECG), which has a dihydroxyl structure in the B ring, does not suppress FcεRI expression in KU812 cells (*24*). Among the five methylated EGCGs, EGCG 4′Me and EGCG 4′4″diMe have two hydroxyl groups in the B ring. These results suggest that the trihydroxyl structure of the B ring is important for EGCG to suppress both FcεRI expression and the ERK1/2 phosphorylation.

Next, we investigated the cell surface binding activity of the five methylated derivatives of EGCG. As a result of SPR analysis, the binding activities of all the methylated derivatives were shown to be lower than that of EGCG (**Figure 4**). Previously, we reported that catechin, epicatechin, and epigallocatechin could not bind to the cell surface of human lung cancer A549 cells (*21*). These findings suggest that the gallate (gallic acid ester) moiety is indispensable for EGCG's binding to the cell surface, and this may be why the strength of binding for the four methylated derivatives that have the methyl ether group in their gallate moiety was lower than that of EGCG. Among the methylated derivatives we tested in this study, only EGCG 4′4″diMe, which has the methyl ether group in both the B ring and the gallate moiety, could not bind to the cell surface.

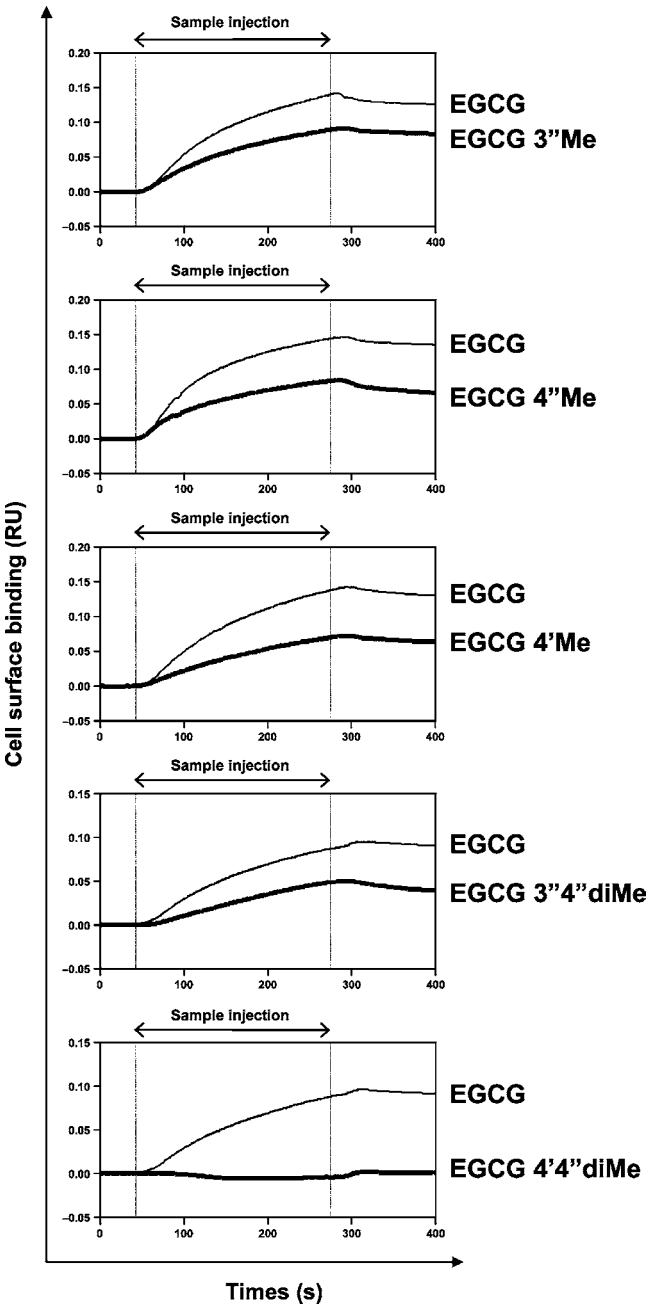

Figure 4. Cell surface binding activities of EGCG and each of the methylated derivatives of EGCG. Cells were immobilized on the sensor chip. The cell surface interaction of each methylated derivative (25 μM) with KU812 cells was measured using the SPR biosensor. The thin solid line and the thick solid line show EGCG and methylated derivatives of EGCG, respectively.

These results suggest that hydroxyl groups on not only the 4″-position in the gallate moiety but also the 4′-position in the B ring are crucial to bind to the cell surface.

Among the four methylated EGCGs that could indeed bind to the cell surface, only EGCG 4′Me was not able to suppress FcεRI expression. These findings suggest that although the binding of EGCG and its methylated derivatives to the cell surface is necessary for initiation of the biological response, binding alone is not sufficient to induce the reduction of ERK1/2 phosphorylation, which in turn may lead to a decrease in FcεRI expression.

In summary, not only the gallate moiety but also the structure of the B ring are involved in the binding of EGCG to the cell

surface. Moreover, the cell surface binding is necessary for EGCG to suppress FcεRI expression and ERK1/2 phosphorylation. However, it is not sufficient to exert both activities.

We recently found that 67LR is the molecular target on the cell surface for EGCG and that EGCG suppresses FcεRI expression through binding to 67LR (*21*, *25*). In addition, we reported that 67LR is located in lipid raft regions and that EGCG localizes in the raft regions (*25*). EGCG 4′Me could bind to the cell surface; however, this derivative did not suppress either FcεRI expression or ERK1/2 phosphorylation. Interestingly, ECG also binds to the cell surface but could not, however, suppress FcεRI expression, and ECG localizes in nonraft regions (*24*). EGCG 4′Me may prefer to bind to nonraft regions rather than to raft regions as well as ECG. In the future, we intend to examine whether the methylated derivatives of EGCG also exert their biological activities via 67 LR.

In this study, we found that the suppressive effect of EGCG 3″Me and EGCG 4″Me, which have been shown to be potent anti-allergic agents (*16*, *17*), on FcεRI expression and their cell binding activities are low compared with those of EGCG. EGCG is known to be unstable and is degraded easily in animal bodies. On the other hand, EGCG 3″Me and EGCG 4″Me are absorbed efficiently and are more stable than EGCG in animal and human plasma (unpublished data), suggesting the reason for the methylated derivatives of EGCG having potent inhibitory activities to allergies *in vivo*, whereas the biological activities *in vitro* are lower than those of EGCG.

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