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Lipid raft associated catechin suppresses the FcεRI expression by inhibiting phosphorylation of the extracellular signal-regulated kinase1/2

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Abstract The major green tea catechin, (−)-epigallocatechin-3-*O*-gallate (EGCG), has a suppressive effect on the expression of the high-affinity IgE receptor FcεRI, which is key molecule in the IgE-mediated allergic reactions. Here we show that EGCG binds to the cell surface and highly associates with plasma membrane microdomains, lipid rafts, on the human basophilic KU812 cells. The disruption of these lipid rafts caused a reduction of the amount of raft-associated EGCG and the FcεRI-suppressive effect of EGCG. We also found that EGCG has an ability to inhibit the phosphorylation of the extracellular signal-regulated kinase1/2 (ERK1/2) and that the ERK1/2 specific inhibitor also reduced FcεRI expression. Moreover, the inhibitory effect elicited by EGCG on ERK1/2 was prevented by disruption of rafts. Thus, these results suggest that the interaction between EGCG and the lipid rafts is important for EGCG's ability to downregulate FcεRI expression, and ERK1/2 may be involved in this suppression signal.

Key words: High-affinity IgE receptor; (−)-Epigallocatechin-3-*O*-gallate; Tea catechin; Lipid raft; Extracellular signal-regulated kinase; KU812

1. Introduction

The high-affinity IgE receptor FcεRI is expressed on the surface of effector cells, such as mast cells and basophils. Crosslinking of allergen specific IgE bound with FcεRI by a multivalent antigen induces activation of these cells and results in the secretion of allergic mediators as well as the induction of cytokine gene transcription [1]. Therefore, FcεRI plays a central role in the induction and maintenance of IgE-mediated allergic responses such as atopic dermatitis, bronchial asthma, allergic rhitis, and food allergy. The FcεRI molecule on these cells is a tetrameric structure of one α chain, one β chain, and two disulfide-linked γ chains. In addition, a trimeric form of FcεRI which lacks the β chain is found in human. Among the three subunits forming FcεRI, the α chain is the specific component of FcεRI that mostly extends out to the extracellular region and directly binds to IgE. Analysis of α chain-deficient mice demonstrated that IgE was unable to bind to the cell surface of mast cells, thereby inabling the induction of degranulation through IgE binding [2]. Thus, it is expected that the downregulation of FcεRI expression in mast cells and basophils may lead to the attenuation of the IgE-mediated allergic symptoms.

We recently found that the major catechin in green tea, (−)-epigallocatechin-3-*O*-gallate (EGCG), was able to decrease FcεRI expression and negatively regulate basophil activation [3,4]. The biological and pharmacological properties of EGCG, including anti-oxidative and cancer-preventive effects, have been reported [5–7]. The modulation of epidermal growth factor receptor function, protein kinase C activation, mitogen-activated protein kinase (MAPK) cascades were thought to be the most interesting targets to evaluate the effect of EGCG [8–10]. It has been suggested that EGCG and its derivatives exhibit biological activities through interactions with the cellular membranes [11]. The study, using the liposome system, on the interaction of tea catechins with the lipid bilayers implicates that the affinity of catechins for the lipid bilayers may be responsible for various kinds of actions [12]. Further investigation about the cellular interaction of EGCG may contribute to the elucidation of the mechanism of how EGCG can decrease FcεRI expression.

Recently, plasma membrane microdomains referred to as 'lipid rafts' have received much attention as potential regulators and organizing centers for signal transduction and membrane traffic pathways [13]. These microdomains have been characterized as sphingolipid/cholesterol-rich domains in the plasma membranes. Lipid rafts have also been shown to interact with various kinds of extracellular factors including cytokines and growth factors, neuroproteins, and viruses [14–17]. However, little is known about the interaction of these microdomains with low molecular compounds such as tea catechins.

In this report, we have shown that EGCG binds to the cell surface and interacts with the plasma membrane microdomain, lipid rafts in the human basophilic KU812 cells. In addition, our results suggested that the inhibition of extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylation, which is the member of MAPK family, is involved in the ability of EGCG to suppress the expression of FcεRI, and further lipid rafts may play an important role in downregulatory signaling.

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Abbreviations: CTXB, cholera toxin B; EGCG, (−)-epigallocatechin-3-*O*-gallate; ERK1/2, extracellular signal-regulated kinase1/2; FcεRI, high-affinity IgE receptor; HPLC, high-performance liquid chromatography; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; RT-PCR, reverse transcription-polymerase chain reaction; SPR, surface plasmon resonance

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2. Materials and methods

2.1. Reagents

EGCG was purchased from Kurita Water Industries LTD (Tokyo, Japan). Protein A Sepharose beads were purchased from Amersham Pharmacia Biotech (UK). Mouse anti-human FcεRI α chain antibody CRA-1 was obtained from Kyokuto seiyaku (Tokyo, Japan). Mouse IgG2b, used as negative control, was bought from Dako (A/S, Denmark). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody was purchased from Protos Immunoresearch (Burlingame, CA, USA). Both anti-phosphorylated p38 MAPK and c-jun N-terminal kinase (JNK) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse anti-phosphorylated ERK1/2 antibody and rabbit anti-ERK1/2 antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse IgG antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody, and HRP-conjugated anti-rabbit antibody were obtained from Zymed Laboratories, Inc. (San Francisco, CA, USA) and ICN Pharmaceuticals, Inc. (Aurora, OH, USA), respectively. Methyl-β-cyclodextrin (MβCD), FITC-conjugated cholera toxin B (CTXB), and HRP-conjugated CTXB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MAPK specific inhibitors, PD98059, SB203580 and SP600125, were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA).

2.2. Cell culture and stimulation

KU812 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were cultured in serum-free RPMI-1640 medium with or without EGCG or MAPK specific inhibitors. MAPK inhibitors were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO being 0.1%. This concentration of DMSO had no effect in any of the experiments. For the cholesterol depletion study, cells were treated in a lipid-free medium with 5 mM MβCD for 45 min at 37°C. After washing, the cells were used in the following experiments. Cellular cholesterols were measured by using a commercial kit (Wako Pure Chemical Industries, Osaka, Japan). We checked the cell viability of KU812 cells treated with or without MβCD by trypan-blue exclusion methods.

2.3. Flow cytometric analysis of the cell surface expression of FcεRI

The cell surface expression of FcεRI was assessed by flow cytometry as previously described [3]. In brief, cells were incubated with the anti-FcεRI α chain antibody CRA-1. Then cells were exposed to the FITC-conjugated anti-mouse IgG and subjected to flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, CA, USA). As a negative control, the mouse subclass-matched IgG2b antibody was used. The extent of FcεRI expression is represented as the mean fluorescence intensity of CRA-1, and the value is indicated in each panel (Fig. 3). The vertical line in the figure indicates the peak point for CRA-1 in non-treated cells.

2.4. Binding analysis using surface plasmon resonance (SPR) biosensor

Analysis of the interaction between catechin derivatives and KU812 cells was performed using the SPR biosensor SPR670 (Nippon Laser and Electronics Lab., Nagoya, Japan). KU812 cells were immobilized on the sensor chip and then this chip was equilibrated in SPR running buffer, phosphate-buffered saline (pH 7.4, 30 μl min^{-1}). EGCG was diluted at 0 or 50 μM in SPR running buffer in 60 μl injection volumes and at a flow rate of 30 μl min^{-1}. Binding was measured at 25°C for 2 min, followed by dissociation. The value of the angle in Fig. 2 corresponded with the binding strength.

2.5. Purification of raft fractions

After treatment of KU812 cells with or without 50 μM EGCG for 24 h, raft fractions were prepared by cell lysis using 1% Triton X-100 followed by sucrose gradient fractionation as described previously [18]. One-ml fractions were collected from the top of the gradient [designated fractions number 1 (top) through 12 (bottom)]. For experiments that involved pooling of fractions, low-buoyant density fractions 4–6 were designated raft fractions and high-buoyant density fractions 11–12 were designated non-raft fractions. Protein concentration of each fraction was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Pooled raft and non-raft fractions were then internally normalized for protein content.

2.6. Quantitation of EGCG

The level of EGCG in raft and non-raft fractions was determined by high-performance liquid chromatography (HPLC) with a coulometric array detector (Model 5600 CoulArray, ESA Inc., Chelmsford). In brief, 200 μg of raft and non-raft fractions were extracted by ethyl acetate twice. The combined ethyl acetate solutions were added to a 20% ascorbic acid solution, and dried under nitrogen gas and then redissolved in a 50% acetonitrile aqueous solution. The resultant solution was injected onto the HPLC with TSK gel ODS 80Ts reversed-phase column (4.6×250 mm, Tosoh, Tokyo, Japan). The column was eluted at 30°C with 0.1 M NaH_2PO_4 buffer (pH 2.5) containing 0.1 mM EDTA·2Na–acetonitrile (87:13) at a flow rate of 0.6 ml min^{-1}. The eluent was monitored electrochemically at an applied potential of +660 mV versus Ag/AgCl.

2.7. Immunoprecipitation and immunoblot analysis

For immunoprecipitation, KU812 cells were cultured with 10 μM PD98059 or 25 μM EGCG for 24 h under serum-free conditions, and then the cells were lysed as previously described [3]. Whole cell lysates were incubated with protein A Sepharose beads bound with the anti-FcεRI α chain antibody CRA-1 for 4 h at 4°C. The beads were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer. After centrifugation, supernatants were subjected to immunoblot analysis. The immunoprecipitates were loaded onto an 8% SDS–PAGE gel, and electrophoresis was done under reducing conditions. The samples were then electrotransferred onto a nitrocellulose membrane. The membrane was probed for the FcεRI α chain using the anti-FcεRI α chain antibody CRA-1. The secondary antibody used was the HRP-conjugated anti-mouse IgG, and detection was done using the ECL kit (Amersham Pharmacia Biotech). For examination of MAPK, the cells were treated with 25 μM EGCG or 10 μM PD98059 for 3 h, and then lysed. The whole lysate were resuspended in SDS–PAGE buffer and subjected to 10% SDS–PAGE and immunoblotted using the anti-phosphorylated ERK1/2, p38 MAPK, or the JNK antibody and the HRP-conjugated anti-rabbit IgG antibody. For detection of total ERK1/2, the same filter was blotted again with anti-ERK1/2 antibody. The detection of β-actin was simultaneously performed as a gel loading control. To confirm the location of lipid rafts in recovered and pooled sucrose gradient fractions, both low- and high-buoyant density fractions were subjected to 10% SDS–PAGE and immunoblotted using CTXB subunit conjugated to HRP and ECL systems.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis

Total RNA was isolated using the TRIZol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed. The resultant cDNA samples were subjected to 13 cycles of PCR amplification in the presence of specific sense and antisense primers. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was amplified as an internal control. Temperatures were 94°C for denaturation, 60°C for annealing, and 72°C for polymerizations. Sequences for the PCR primers are as follows: for the FcεRI α chain: sense 5′-CTTAGGATGTGGGTTCAGAAGT-3′, and antisense 5′-GACAGTGGAGAATACAAATGTCA-3′; for the FcεRI γ chain: sense 5′-TAGGGCCAGCTGGTGTTAATGGCA-3′, and antisense 5′-GATGATTCCAGCAGTGGTCTTGCT-3′; for G3PDH: sense 5′-GCTCAGACACCATGGGGAAGGT-3′, and antisense 5′-GTGGTGCAGGAGGCATTGCTGA-3′. The amplified PCR products were subjected to agarose gel electrophoresis, transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech) and hybridized with fluorescein-labeled probes specific for FcεRI α and γ chains and G3PDH. The hybridized probe was detected using the Gene Images detection kit (Amersham Pharmacia Biotech).

3. Results

3.1. Interaction between EGCG and plasma membrane microdomain lipid rafts

To elucidate which interactions of EGCG with the cells was responsible for FcεRI-suppressive effect, the involvement of plasma membrane microdomains, lipid rafts, on EGCG function was investigated. The role of lipid rafts in FcεRI-ex-

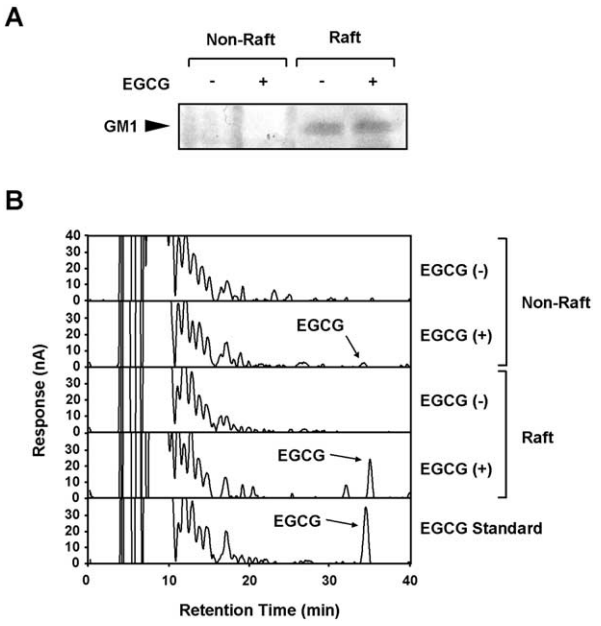

Fig. 1. HPLC chromatograms of EGCG in raft and non-raft fractions isolated from KU812 cells. A: The cells treated with or without EGCG were lysed with cold 1% Triton X-100 followed by a sucrose gradient centrifugation. The low-buoyant density fractions were designated raft fractions and the high-buoyant density fractions were designated non-raft fractions. These fractions were subjected to SDS–PAGE and transferred to nitrocellulose membranes, and GM1-containing lipid rafts were determined using CTXB subunit conjugated to HRP. B: Each fraction mentioned above was subjected to HPLC analysis, and the chromatograms are shown. The retention time for EGCG was confirmed by using the EGCG standard.

pressed cells such as basophils has mostly been analyzed in rat basophilic cell line, RBL-2H3, and the cells have been shown to contain rafts as defined by the resistance of pool of membrane components to extraction by cold Triton X-100 [19–21]. Thus, human basophilic KU812 cells were lysed using cold Triton X-100 followed by a sucrose gradient centrifugation. The low- and high-buoyant density fractions were separately pooled and designated as raft and non-raft fractions, respectively. Because RBL-2H3 cells have been known to express the raft component such as GM1 ganglioside and CTXB subunit has been used to detect this GM1 [20,22], both fractions were analyzed by CTXB subunit blotting. Fig. 1A shows that the raft marker, GM1 ganglioside, was indeed recovered in raft fraction, demonstrating that this fraction does contain lipid rafts isolated from KU812 cells. We also confirmed that the level of GM1 was not affected upon a 24-h treatment with EGCG in both fractions. In addition, EGCG did not affect the cellular level of GM1 over an incubation of 24 h (data not shown). For elucidating the interaction of EGCG with the lipid rafts, the amount of EGCG in the lipid rafts was directly measured by HPLC analysis. The retention time of EGCG was 33 min, and a EGCG peak was not observed in either the raft or non-raft fractions from the cells treated without EGCG (Fig. 1B). On the other hand, in both fractions from EGCG-treated cells, a EGCG peak at the retention time of 33 min was detected. Interestingly, the level of EGCG in the raft fraction was higher than the non-raft fraction, indicating that EGCG does not homogenously bind to the membrane of the cells, but interacts with the cells in a heterogeneous fashion. This suggests that these lipid rafts may play a possible role in the interaction of EGCG with the cells.

3.2. Effect of raft integrity on EGCG binding

To explore a potential role of lipid rafts in EGCG binding, a study of raft integrity was performed using MβCD, a cholesterol-removing agent that disturbs raft function [23]. It has been reported that the treatment with 5 mM MβCD for 30 or 45 min led to 60% extraction of cellular cholesterol in RBL-2H3 cells [19,24]. Treatment with 1 mM MβCD for 45 min demonstrated a slight but significant decrease in the amount of total cholesterol in KU812 cells (Fig. 2A). At a concentration of 5 mM, a reduction of 50% was seen. None of the doses used affected cell viability. The cholesterol lowering effect of MβCD was sustained for a period of at least 24 h (data not shown). Because a raft integrity defect has been known to be induced by a decrease in the amount of cellular cholesterol, these findings may demonstrate that a raft integrity defect was sustained within 24 h after MβCD treatment.

For investigating the effect of lipid rafts disruption on the interaction of EGCG with KU812 cells, we measured the cell surface binding of EGCG to the MβCD-pretreated KU812 cells using a SPR biosensor. This SPR technique has been increasingly used for a real-time analyses of the binding between solubilized molecules and molecules immobilized on the surface of a biosensor chip without any labeling by changes in the refractive index of a biospecific surface [25]. In this binding analysis, the SPR signal has a characteristic behavior as

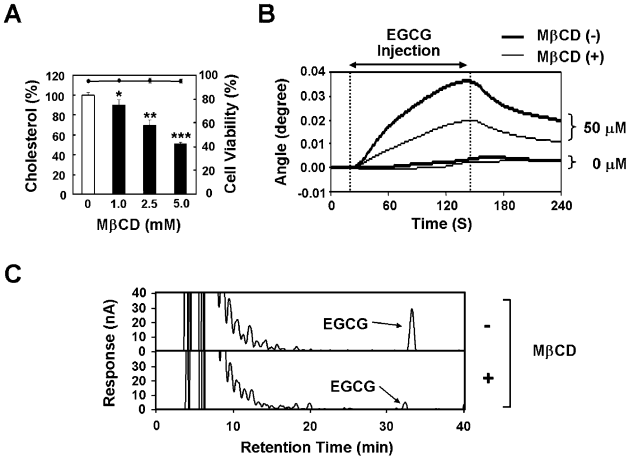

Fig. 2. Effect of cholesterol depletion on the interaction between EGCG and KU812 cells. A: KU812 cells were treated with MβCD at the indicated concentrations for 45 min. Then, the total cellular cholesterol amount and cell viability were measured. Each data value is expressed as mean ± S.D. ($n = 3$). Values are shown as relative to 0 mM data (%) and asterisk marks denote significant differences from the control at each condition at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively. Data is presented as the relative cholesterol amount or cell viability to the control value. B: KU812 cells were pretreated with or without MβCD, and then were fixed on the sensor chip. The cell surface interaction of EGCG with immobilized KU812 cells was measured using a SPR biosensor. EGCG was injected at a concentration of 0 or 50 μM for the indicated interval in the figure. C: After treatment with or without MβCD, KU812 cells were cultured with EGCG for 24 h. Then, the cells were lysed with cold 1% Triton X-100 followed by a sucrose gradient centrifugation. Recovered raft fractions were subjected to HPLC analysis, and the chromatograms are shown.

follows: The elevation of the SPR signal (the angle value) was observed immediately after the injection of the ligands. After the termination of the ligand exposure, the perfusion buffer was changed to the ligand-free running buffer, and the SPR signal was reduced by the dissociation of ligands bound the surface of the immobilized molecules, and the signal converged to a constant level. On the other hand, this technique has been also applied to the analysis of secondary signaling reactions induced by the ligand exposure to the immobilized living cells [26]. In this case, the increase in the SPR signal was observed even after the ligand exposure, and the signal finally returned to the basal level. In the present study, SPR experiments showed that the SPR signal significantly increased by the injection of EGCG, and the SPR signal was reduced just after the termination of the ligand exposure (Fig. 2B). The SPR signal did not return to the base level at even 60 min after exposure (data not shown). This signal pattern appears to be similar to the case of binding signals rather than secondary signaling reactions of the cells. In addition, the signal pattern was obtained in not only the living cells but also the dead cells (data not shown). These results suggested that the SPR signal of EGCG may be due to a result of the binding to the plasma membrane. In the study on a disruption of lipid rafts, the treatment with MβCD clearly decreased the binding of EGCG, and this treatment also led to a reduced amount of EGCG in the raft fraction isolated from MβCD-treated cells (Fig. 2C). These results suggest that lipid rafts may be related to the interaction between EGCG and the cell surface of KU812 cells.

3.3. Effect of raft integrity on the ability of EGCG to suppress FcεRI expression

To further examine the effect of MβCD on the suppression of FcεRI expression by EGCG, after MβCD treatment, KU812 cells was incubated with EGCG for 24 h under serum-free conditions. The cell surface expression of FcεRI was measured by flow cytometric analysis using the FcεRIα chain antibody CRA-1 (Fig. 3A). In the absence of MβCD, EGCG-treated cells showed a 32% (15.2/22.4) decrease in the value of mean fluorescence intensity compared to the non-treated cells, but pretreatment with MβCD reduced this suppressive effect from 32% to 16% (20.4/24.2). Furthermore, the amount of total cellular FcεRI α chain was reduced upon treatment with EGCG, but this suppressive effect was lowered by MβCD pretreatment (Fig. 3B). These results indicated that the ability of MβCD to abrogate the suppressive effect of EGCG on the FcεRI cell surface expression may be due to the abrogation of the reduction of total cellular FcεRI α chain expression. Taken together, this suggests that lipid rafts play an important role in the EGCG-induced suppression of FcεRI expression.

3.4. Involvement of ERK1/2 in the suppressive effect of EGCG on FcεRI expression

EGCG have been known to exert cancer-preventive effects, and tumor-associated protein kinases MAPK have been suggested to be possible targets for actions of EGCG [27–29]. Thus, for elucidating the molecule which mediates the reduction of FcεRI expression by EGCG, we examined the involvement of MAPK. After a 25 μM EGCG treatment for 3 h, the level of phosphorylated MAPKs were measured by immunoblot analysis. EGCG clearly inhibited ERK1/2 phosphorylation, slightly reduced the phosphorylation of JNK, but did not affect p38 MAPK activity (Fig. 4A). We further examined the effect of specific inhibitors to the three MAPKs (PD98059 (ERK1/2), SB203580 (p38 MAPK), or SP600125 (JNK)) at a concentration of 10 μM for 24 h on the FcεRI expression. Flow cytometric analysis showed that among the MAPK inhibitors, a decrease of the cell surface expression of FcεRI was observed only in PD98059-treated cells, whereas no decrease in the level of FcεRI expression upon treatment with the other MAPK inhibitors was found (Fig. 4B). These results suggested that the downregulation of ERK1/2 activity is the important event for decreasing FcεRI expression.

We also examined the effect of PD98059 on the level of the total cellular protein and mRNA expression of FcεRI α chain. Both protein and mRNA levels of FcεRI α chain decreased upon treatment with PD98059 (Fig. 4C,D). In addition, the FcεRI γ mRNA levels in PD98059-stimulated cells was shown to be significantly reduced. These results suggested that the suppressive effect of PD98059 on the cell surface expression of FcεRI is related to the downregulation of the expression of

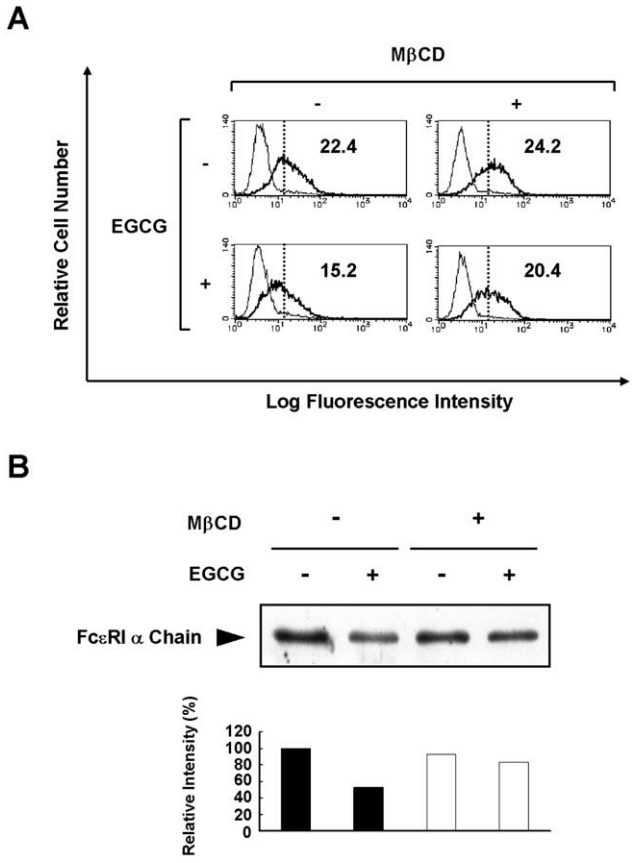

Fig. 3. MβCD pretreatment prevents EGCG from suppressing FcεRI expression. A: After treatment with 5 mM MβCD for 45 min, KU812 cells were stimulated with EGCG at 25 μM for 24 h. Then, the cells were stained with CRA-1 (solid line) or mouse IgG2b (thin line) as an isotype-matched negative control. The fluorescence intensity was determined using the FACSCalibur. The value indicated in the figure is the mean fluorescence intensity of CRA-1. The vertical line in the figure indicates the peak point for CRA-1 in the non-treated cells. B: MβCD-pretreated (+) or non-treated (−) KU812 cells were cultured with (+) or without (−) 25 μM EGCG under serum-free conditions for 24 h. Immunoblot analysis was performed using the anti-FcεRI α chain antibody CRA-1.

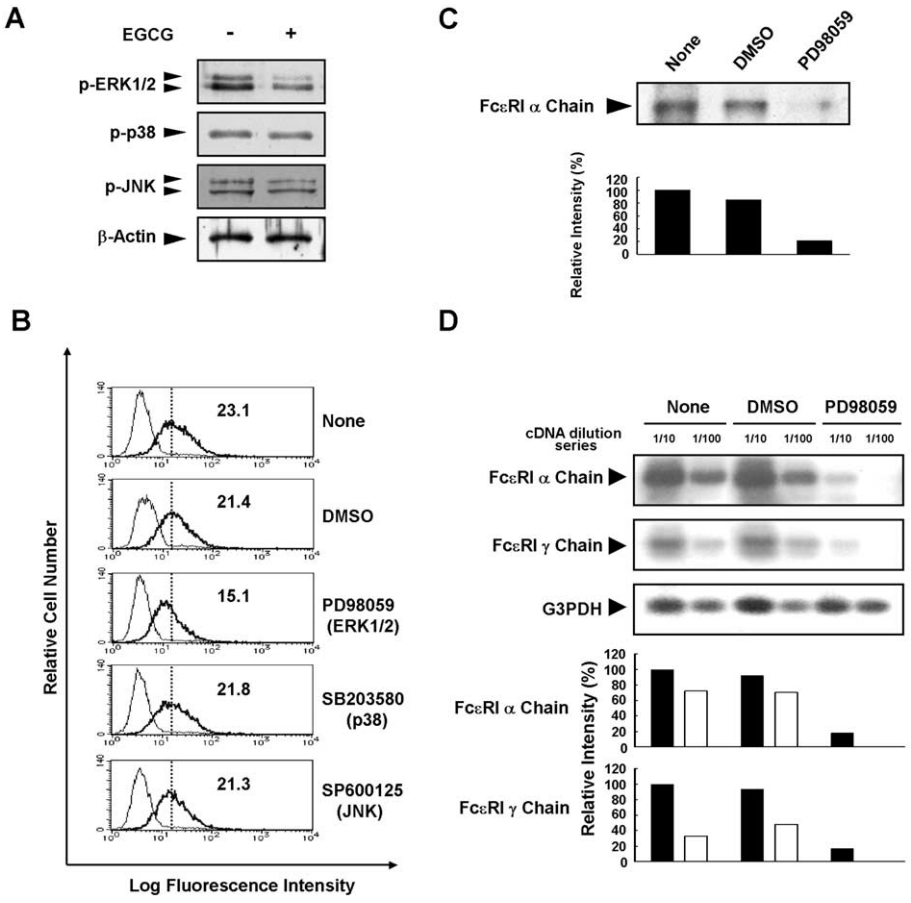

Fig. 4. Effect of MAPK specific inhibitors on the cell surface expression of FcεRI. A: KU812 cells were treated with 25 μM EGCG for 3 h. Each MAPK was separated on a 10% SDS–PAGE and immunoblotted with the anti-phosphorylated ERK1/2, p38 MAPK, and JNK antibodies. The detection of β-actin was simultaneously performed as a gel loading control. B: KU812 cells were stimulated with each MAPK specific inhibitor at 10 μM for 24 h under serum-free conditions. Then, the cells were stained with CRA-1 (solid line) or mouse IgG2b (thin line) as the isotype-matched negative control. The fluorescence intensity was determined using the FACSCalibur. The value indicated in the figure is the mean fluorescence intensity for CRA-1. The vertical line in the figure indicates the peak point for CRA-1 in the non-treated cells. C: KU812 cells were treated with 10 μM PD98059 for 24 h. Immunoblot analysis was performed using the anti-FcεRI α chain antibody CRA-1. D: After treatment with 10 μM PD98059 for 24 h, total RNA was isolated from the cells. FcεRI α, γ and G3PDH mRNA were analyzed by RT-PCR. Southern blotting using specific probes for FcεRI α, γ or G3PDH was performed to assess the PCR products.

FcεRI α and γ chain genes. Moreover, these results and the fact that EGCG was able to decrease the mRNA expression of both FcεRI α and γ chains as previously reported [3] suggest a possibility that the FcεRI-suppressive effect of PD98059 may mimic EGCG.

3.5. *Disruption of lipid rafts cancel the inhibition of the ERK1/2 phosphorylation by EGCG*

To clarify whether or not the reductive action of ERK1/2 phosphorylation by EGCG is transduced through the interaction with the lipid rafts, after treatment with MβCD, the cells were cultured with EGCG for 3 h and the level of phosphorylated ERK1/2 was determined by immunoblotting. This analysis revealed that EGCG reduced the level of ERK1/2 phosphorylation in the cells without MβCD, but pretreatment with MβCD canceled this inhibitory effect (Fig. 5). Since the total level of ERK1/2 was not affected by MβCD pretreatment, lowering the effect of EGCG was not responsible for enhancing total amounts of ERK1/2 by MβCD. Therefore, these results indicated that lipid rafts were the principal location for transducing the suppressive effect of EGCG on ERK1/2 phosphorylation.

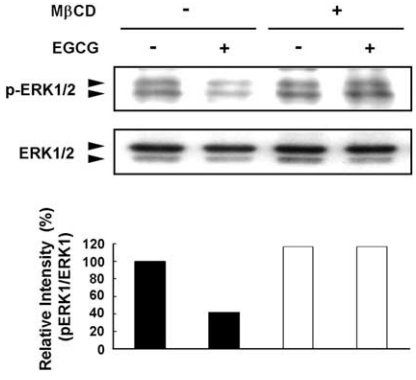

Fig. 5. MβCD pretreatment cancels the inhibitory effect of EGCG on the ERK1/2 activation. After treatment with 5 mM MβCD for 45 min, KU812 cells were stimulated with 25 μM EGCG for 3 h. ERK1/2 was separated on a 10% SDS–PAGE and immunoblotted with the anti-phosphorylated ERK1/2 antibody. Shown in the lower panel are protein levels from the same filter blotted again with the anti-ERK1/2 antibody.

4. Discussion

EGCG has been found to inhibit epidermal growth factor- or platelet-derived growth factor-mediated tumor cell growth by reducing the autophosphorylation of their receptors [8], and both of these receptors were observed in lipid rafts [14,15]. The formation of senile plaques containing the β amyloid peptide (Aβ) derived from the amyloid precursor protein (APP) is an invariant feature of Alzheimer's disease. It has been demonstrated that APP interacts with lipid rafts and which are critically involved in regulating Aβ generation [30]. Aβ has been known to be toxic to neurons in rat primary hippocampal cultures [31], and such neurotoxicity have been shown to be attenuated by treatment with EGCG [32]. These circumstances have fueled interest in the role of lipid rafts as a platform for researching the function of EGCG, but there has been no direct data proving the relationship of EGCG with lipid rafts.

In this study, more of EGCG was found in the raft fraction (Triton X-100-resistant pool) than the non-raft fraction, indicating that EGCG is sensitive to cholesterol depletion. A cholesterol-depleted study showed a remarked reduction of EGCG in the raft fraction. Although total cellular level of EGCG was decreased upon treatment with MβCD, there was little change in the amount of EGCG in the non-raft fraction after cholesterol depletion (data not shown). The measurements of EGCG contents have been repeatedly performed and representative data have been shown. These observations showed that cholesterol depletion increased in the partitioning rate of EGCG from the raft fraction to the non-raft fraction. These facts may suggest a preference of EGCG for a certain lipid phase in the original membrane, but it might also reflect partitioning of EGCG during the Triton X-100 incubation. Although results of HPLC analysis may show the amount of EGCG in all cellular membrane, not just plasma membrane, SPR analysis directly reflects the interaction of EGCG with the cell surface, that is, plasma membrane. The SPR signal shown here was reduced by 50% after cholesterol depletion, indicating that the amount of EGCG bound to the cell surface, plasma membrane, of the immobilized cells decreases by half of intact level. The extent of EGCG's binding to plasma membrane was indeed correlated with the level of EGCG in the raft fraction. In addition, the cholesterol depletion did not influence the amount of phospholipid in the immobilized cells (data not shown). These results suggest that the reduced SPR signal after cholesterol depletion does not result from a reduction of phospholipid, but it may be due to a decrease in the amount of EGCG bound to lipid rafts on the cell surface. This raised a possibility that the target of EGCG may be not only lipid components such as cholesterol, but also a membrane protein in lipid rafts on the cell surface. Now, the investigation for the cell surface molecule acting as an EGCG receptor, which can mediate the FcεRI-suppressive effect, is proceeding.

The FcεRI of the basophilic RBL-2H3 cells in resting state has been reported to be not associated with lipid rafts, and the receptor aggregation induces a weak association with rafts [20]. Our results that the expression of FcεRI in resting state was not affected by cholesterol depletion may suggest a possibility that non-aggregated FcεRI does not associate with lipid rafts on the surface of human basophilic KU812 cells. The cholesterol depletion also induced the inability of EGCG to suppress the FcεRI expression. This treatment did not affect the FcεRI expression, but caused a significant reduction of the amount of EGCG in the lipid rafts, which may be due to a lowered binding of EGCG to the cells. Therefore, this reduction of EGCG bound to lipid rafts may cause the diminishing of the FcεRI-suppressive signal. However, it is not able to exclude the possibility that the lowering of cholesterol during 24 h may have induced many changes in the cell that are unrelated to the structure and function of lipid rafts, and further investigations are necessary. Taken together, these results suggest that lipid rafts play a critical role in EGCG's ability to suppress FcεRI expression. As far as we know, this finding is the first evidence proving direct linkage of EGCG functions with the lipid rafts.

Recently, EGCG has been reported to have a modulatory effect on ERK1/2 as well as other MAPKs, p38 MAPK and JNK, cascades [10,29,33]. However, neither a p38 MAPK nor JNK specific inhibitor directly affected the cell surface expression of FcεRI, nor the inhibitors were able to cancel EGCG's ability to suppress FcεRI expression (data not shown). This indicates that the decrease in FcεRI expression by EGCG was independent of the p38 MAPK and JNK cascades. On the other hand, our experiments revealed that EGCG had an inhibitory effect on ERK1/2 phosphorylation, and the ERK1/2 specific inhibitor PD98059 suppressed FcεRI expression. This FcεRI-suppressive effect was observed after more than a 12-h treatment similar to EGCG, and further PD98059 clearly reduced the level of phosphorylated ERK1/2 from 3 to 12 h after the addition, which closely mirrored EGCG's ability to induce a decrease in ERK1/2 phosphorylation (data not shown). An incubation of 3 or 6 h with EGCG or PD98059 indeed affected ERK1/2 phosphorylation, but there was no change in the FcεRI expression. PD98059 treatment also lowered the level of FcεRI α and γ mRNA as previously reported for EGCG [3]. These findings suggest that at least a 12-h sustained reduction of ERK1/2 phosphorylation was required for downregulation of the FcεRI expression by EGCG. In mast cells and basophils played a central role in the FcεRI-mediated allergic reaction, the involvement of MAPKs in cytokine production, release of chemical mediators, cell survival and regulation of apoptosis have been indicated [34–36]. However, with regard to FcεRI expression, there is very little information concerning the MAPK signal pathways. On the other hand, the ERK1/2 activation has been reported to depend on lipid rafts [37,38]. In consideration of this report and our observations that EGCG associates with lipid rafts, it is not unexpected that the raft disruption prevents EGCG from inhibiting the ERK1/2 phosphorylation. These findings may also provide new insights, as to the involvement of lipid rafts, into the mechanism for the inhibition of ERK1/2 signaling by EGCG.

In the present study, we observed that the disruption of lipid rafts inhibited the suppressive effect of EGCG on the ERK1/2 activation. This is thought to be direct evidence showing that lipid rafts play a critical role in EGCG's ability to reduce ERK1/2 phosphorylation. In addition, it was speculated that diminishing the cascade signals may be caused by a lowering of the interaction between EGCG with the lipid rafts, resulting in a reduced suppression of FcεRI expression.

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