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The 67 kDa laminin receptor as a primary determinant of anti-allergic effects of *O*-methylated EGCG

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Abstract

Previously we have reported that the *O*-methylated derivative of (−)-epigallocatechin-3-*O*-gallate (EGCG), (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3''Me), possesses anti-allergic activities such as inhibition of histamine release and suppression of the high-affinity IgE receptor (FcεRI) expression. However, the underlying mechanism is still unclear. Recently we have identified the 67 kDa laminin receptor (67LR) as a cell-surface receptor that can mediate biological activities of EGCG. Here we show that the suppression of myosin II regulatory light chain (MRLC) phosphorylation through the cell-surface binding to the 67 LR contributes to the inhibitory effect of EGCG3''Me on the histamine release from the human basophilic KU812 cells. The 67LR also mediated the EGCG3''Me-induced suppression of FcεRI expression by reducing ERK1/2 phosphorylation. These results suggest that anti-allergic effects of EGCG3''Me may be triggered by the inhibition of MRLC or ERK1/2 phosphorylation mediated through the cell-surface 67LR.

Keywords: *O*-Methylated EGCG; 67 kDa laminin receptor; Allergy; Degranulation; Myosin regulatory light chain; FcεRI; ERK1/2

Allergen-specific IgE molecules bind to the high-affinity IgE receptor (FcεRI) on the surface of mast cells or basophils. Clustering of allergens and crosslinking of IgE molecules bound to the FcεRI induces cell activation, and inflammatory mediators such as histamine, cytokines, chemotactic factors, and arachidonic acid metabolites are released. These mediators ultimately cause allergic symptoms including atopic dermatitis, hay fever, and food allergy [1].

Tea (*Camellia sinensis* L.) is one of the most widely consumed beverages in the world. Green tea catechin present in tea leaves is believed to be the compound most responsible for the health benefits attributed to tea. Recently, there is an accumulating body of evidence showing that *O*-methylated forms of (−)-epigallocatechin-3-*O*-gallate (EGCG) have potent inhibitory activities to allergies in vivo [2–4]. A clinical study indicates that intake of 'Benifuuki' green tea, which contains high levels of *O*-methylated EGCGs, is useful in reducing the symptoms of allergic cedar-polliosis [4]. We and our colleagues have found that EGCG and its methylated derivative, (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3''Me), were able to suppress FcεRI expression and histamine release [5–10]. We have also reported that the cell-surface binding of EGCG3''Me to the basophilic cells is involved in the suppressive effect of EGCG3''Me on FcεRI expression [5]. However, the cell-surface-mediated molecular basis for actions of EGCG3''Me remains to be investigated.

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We have previously identified the 67 kDa laminin receptor (67LR) as a cell-surface receptor that mediates the anticancer action of EGCG [11]. This receptor has been shown to be responsible for anti-allergic effects of EGCG [12,13]. We have also found that the inhibition of myosin II regulatory light chain (MRLC) or extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylation plays a critical role in the EGCG-induced degranulation inhibition or suppression of FcεRI expression, respectively [10,13]. In this study, whether these signaling molecules are involved in EGCG3''Me's abilities to downregulate the degranulation or the FcεRI expression was examined. We found that anti-allergic effects of EGCG3''Me are elicited by the cell-surface binding to the 67LR, and demonstrated that the inhibition of either MRLC or ERK1/2 phosphorylation is involved in the 67LR-mediated anti-allergic actions of EGCG3''Me.

Materials and methods

Reagents. EGCG3''Me was prepared from green tea leaves (*Camellia sinensis* L. cv. Benifuuki) according to the method previously reported [2,9]. Anti-phosphorylated MRLC (Thr18/Ser19), anti-MRLC, anti-phosphorylated ERK1/2, and anti-ERK1/2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-human FcεRI α chain antibody CRA-1 was obtained from Kyokuto seiyaku (Tokyo, Japan). Mouse IgG2b was bought from Dako (A/S, Denmark). The anti-67LR antibody (MLuC5) was purchased from NEOMARKERS (Fremont, CA). Mouse IgM antibody was obtained from Zymed Laboratories, Inc. (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody was purchased from Protos Immunoresearch (Burlingame, CA). Catalase, superoxide dismutase (SOD), calcium ionophore A23187, and anti-β-actin and FITC-conjugated anti-mouse IgM antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. KU812 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 mM Hepes buffer. The 67LR-downregulated KU812 cells (67LR-shRNA (+)) were previously constructed using RNA interference (RNAi)-mediated gene silencing [12]. For FcεRI test, the cells were cultured in serum-free RPMI-1640 medium with or without 200 U/mL catalase plus 5 U/mL SOD in the presence or absence of 25 μM EGCG3''Me for 24 h. For histamine test, the cells were suspended in buffer (pH 7.4), containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 0.4 mM NaH_2PO_4, with or without 200 U/mL catalase plus 5 U/mL SOD. Then the cells were treated with or without 25 μM EGCG3''Me for 20 min.

Analysis of cell-surface binding. Analysis of the interaction between EGCG3''Me and KU812 cells was performed using the surface plasmon resonance (SPR) biosensor SPR670 (Moritex Corp., Tokyo, Japan) as previously reported [10]. The cells were immobilized on the sensor chip and then this chip was equilibrated in PBS. EGCG3''Me (25 μM) was added at a flow rate of 30 μl/min. The cell-surface binding was measured at 25 °C for 2 min, followed by dissociation. The value of resonance unit (RU) in the figure corresponded with the binding strength.

Immunoblot analysis. After stimulation, the cells were lysed in cell lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethanesulfonyl fluoride, 2.0 μg/ml aprotinin, and 1 mM pervanadate. Whole cell lysate was incubated at 4 °C for 30 min and then centrifuged at 15,000*g* for 30 min. The supernatant was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. To detect the ERK1/2 or MRLC phosphorylation, immunoblot analysis was performed as previously described [10,13]. The density of each band was quantified using a computer program obtained from the U.S. NIH.

Histamine measurement. The cells treated with or without EGCG3''Me were stimulated with 5 μM A23187 at 37 °C for 20 min. The reaction was terminated by cooling for 5 min at 4 °C. After centrifugation at 300*g* for 5 min, the histamine content of the supernatant or cell lysate was measured by the fluorometric assay.

Flow cytometric analysis. The cell-surface expression of FcεRI or 67LR was assessed by flow cytometry as previously reported [12]. The cells were incubated with the anti-FcεRI α chain antibody CRA-1 or anti-67LR antibody MLuC5. Then cells were exposed to the FITC-conjugated anti-mouse IgG antibody or anti-mouse IgM and subjected to flow cytometry (FACSCalibur; Becton–Dickinson, Sunnyvale, CA). The extent of FcεRI or 67LR expression is represented as the mean fluorescence intensity (MFI) of CRA-1 or MLuC5.

Results

ROS generation-independent biological activities of EGCG3''Me

The activation of mast cells and basophils induces a series of intracellular events including activation of protein tyrosine kinases and elevation of intracellular Ca^{2+} levels [14]. After the Ca^{2+} influx, the remodeling of actin–myosin cytoskeleton is induced, which lead to release granule contents including histamine [15,16]. EGCG has flavan-3-ol structure with A and B rings and a galloyl group, and EGCG3''Me is the *O*-methylated form of the 3''-position (OH) of the galloyl moiety in EGCG (Fig. 1A). Our previous reports demonstrated that EGCG3''Me inhibits degranulation induced by the elevated concentration of cytosolic Ca^{2+} using the calcium ionophore A23187 in human basophilic KU812 cells [6]. However, the underlying mechanism still remains unclear. It is generally known that polyphenolic compounds generate the reactive oxygen species (ROS) such as H_2O_2 and superoxide radical in many cell culture systems, and ROS can exert biological effects on the cells [17–20]. Results from in vitro studies with polyphenolic compounds, especially at high concentrations, must be interpreted with caution to clearly distinguish between the ROS generation-dependent and -independent effects. Both catalase and SOD have been often used to abolish the polyphenolic compound-induced ROS generation [19]. We examined whether both enzymes change the effect of EGCG3''Me on the histamine release (Fig. 1B). The addition of catalase (200 U/ml) plus SOD (5 U/ml), these concentrations have been reported to be effective to diminish the ROS generation induced by catechins [19], could not alter the EGCG3''Me-induced inhibition of histamine release from KU812 cells. This suggested that the inhibition of histamine release is caused by the ROS-independent effect of EGCG3''Me.

We have previously reported that EGCG3''Me can inhibit the histamine release as well as suppress the FcεRI expression [6,8]. To examine whether suppressive effect of EGCG3''Me on the FcεRI expression is due to the ROS-

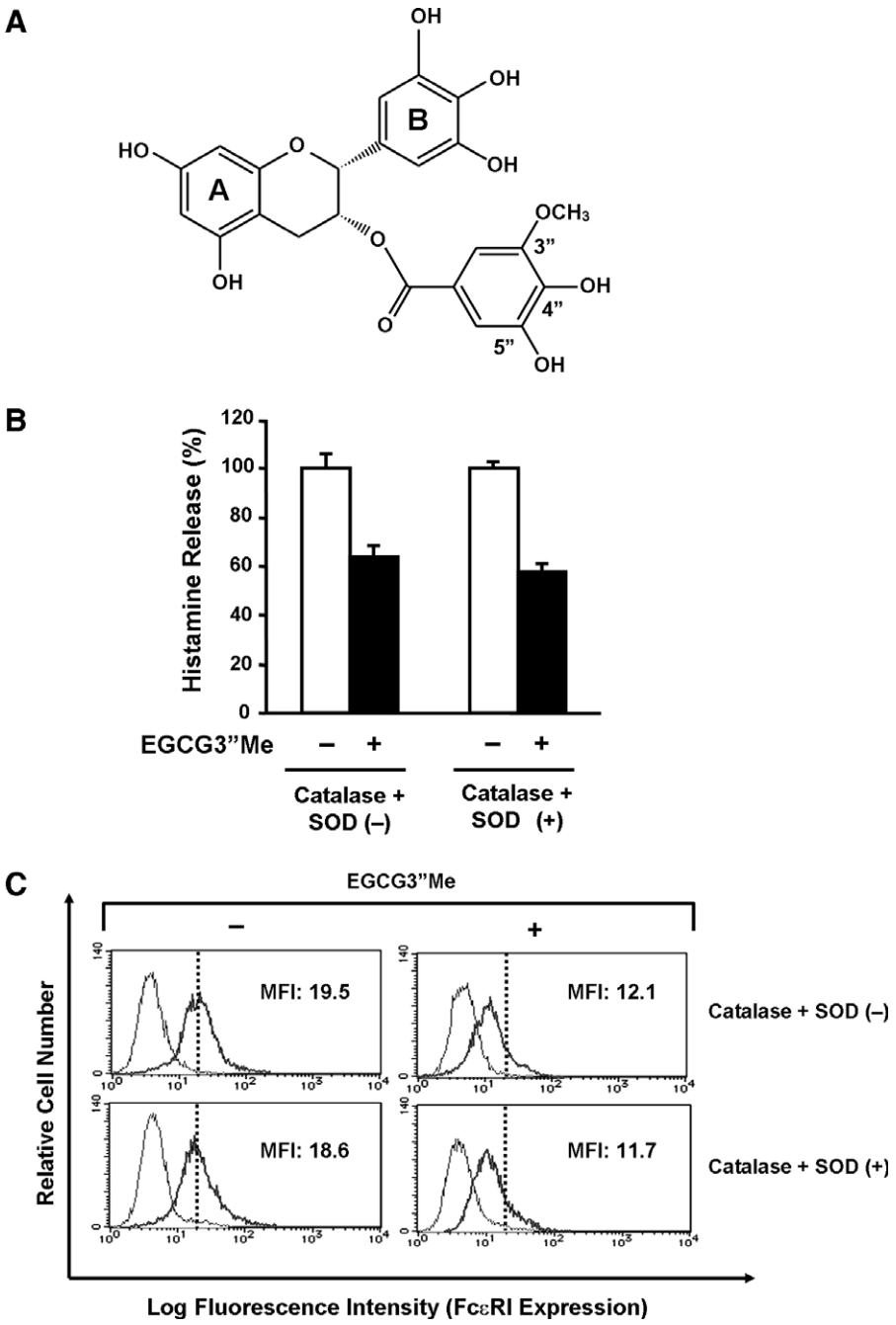

Fig. 1. The effect of catalase plus SOD on two anti-allergic actions of EGCG3''Me. (A) Chemical structure of EGCG3''Me. (B) After treatment of KU812 cells with 25 μM EGCG3''Me for 20 min in the presence or absence of 200 U/ml catalase plus 5 U/ml SOD, the cells were stimulated with 5 μM A23187 for 20 min. The concentration of the released histamine was measured by a fluorometric assay. Results are expressed as means ± SD ($n = 3$). Data are presented as a relative histamine release (%) to the value of EGCG3''Me (−) at each condition. (C) KU812 cells were stimulated with 25 μM EGCG3''Me for 24 h in the presence or absence of catalase plus SOD. Then, the cells were stained with CRA-1, and the fluorescence intensity was determined. The vertical line in figure indicates the peak point for CRA-1 in EGCG3''Me (−) cells.

independent action, we added both catalase and SOD to the culture medium of KU812 cells. As shown in Fig. 1C, there was no alteration of the ability of EGCG3''Me to suppress the FcεRI expression by the addition of two enzymes. This result indicates that the ROS generation-independent mechanism is involved in the suppressive effect of EGCG3''Me on the FcεRI expression.

Involvement of the 67LR in the cell-surface binding of EGCG3''Me

Our previous works demonstrated that the cell-surface binding of EGCG and its methylated derivative is involved in their biological activities [5,10–13,21]. Furthermore, the 67LR has been shown to be the primary target on the cell surface for EGCG to exert its effects. Although there are

several similar properties between EGCG and EGCG3''Me, the molecular target of EGCG3''Me has not been reported at all. To investigate the possibility whether the binding of EGCG3''Me to the cell-surface 67LR is involved in anti-allergic effects of EGCG3''Me, the 67LR-downregulated KU812 cells were used (Fig. 2A), whose cells were previously constructed using RNAi-mediated gene silencing [12]. We examined the binding of EGCG3''Me to the cell surface of the 67LR-downregulated KU812 cells. SPR assay revealed that EGCG3''Me was able to bind the cell surface, and the RNAi-mediated knockdown of 67LR expression resulted in a decreased binding of EGCG3''Me (Fig. 2B), suggesting the involvement of the 67LR in the cell-surface binding of EGCG3''Me.

EGCG3''Me-induced degranulation inhibition is exerted by modulating MRLC phosphorylation through its binding to the cell-surface 67LR

For elucidating the relationship between the 67LR-dependent cell-surface binding of EGCG3''Me and its ability to suppress the histamine release, 67LR-shRNA cells were pretreated with EGCG3''Me in the presence of catalase plus SOD, and then the cells were stimulated with the calcium ionophore A23187. The concentration of the released histamine was measured using a fluorometric assay. As shown in Fig. 3A, the knockdown of 67LR expression significantly abrogated the inhibitory effect of EGCG3''Me on degranulation, implicating that the binding of EGCG3''Me to the cell-surface 67LR may contribute to exert the effect of degranulation inhibition. Thr18/Ser19 phosphorylation of MRLC, which is a component of myosin cytoskeleton, has been reported to be temporally correlated with degranulation in the basophilic cells, and the inhibition of MRLC phosphorylation has been shown

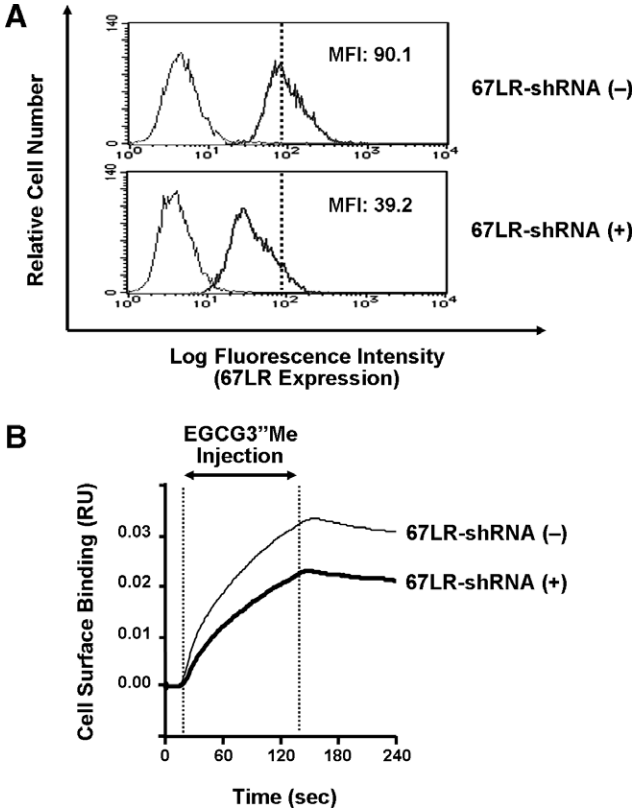

Fig. 2. The 67LR-mediated cell-surface binding of EGCG3''Me to KU812 cells. (A) The cell-surface expression of 67LR in the 67LR-shRNA (−) or (+) cells was measured by flow cytometric analysis using the anti-67LR antibody MLuC5. The vertical line in figure indicates the peak point for MLuC5 in 67LR-shRNA (−) cells. (B) The 67LR-shRNA (−) or (+) cells were fixed on the sensor chip. The cell-surface binding of EGCG3''Me to immobilized KU812 cells was measured using the SPR biosensor. EGCG3''Me was injected for the indicated time interval in the figure.

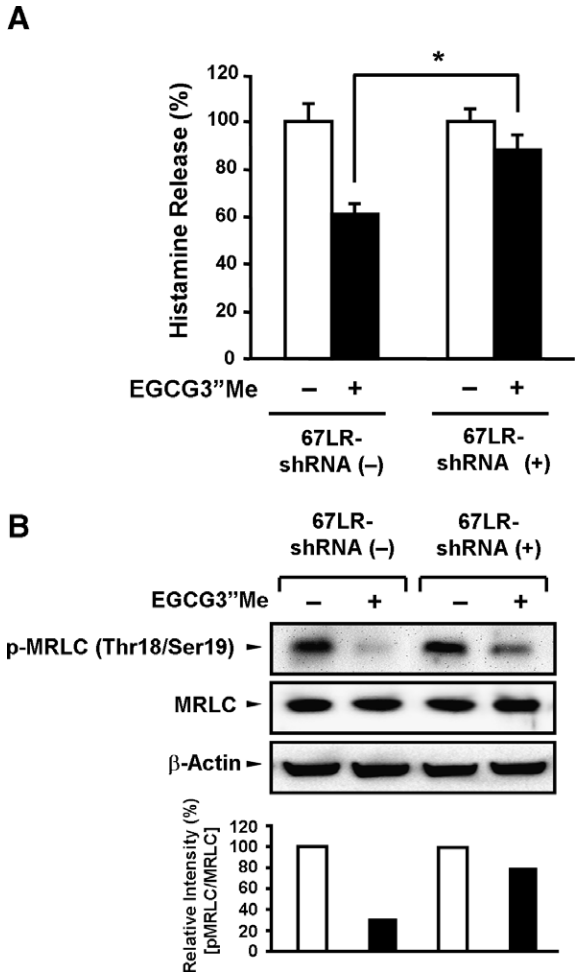

Fig. 3. The involvement of the 67LR in the inhibitory effect of EGCG3''Me on the histamine release. (A) After treatment of the 67LR-shRNA (−) or (+) cells with 25 μM EGCG3''Me for 20 min in the presence of catalase plus SOD, the cells were stimulated with 5 μM A23187 for 20 min. The concentration of the released histamine was measured by a fluorometric assay. Results are expressed as means ± SD ($n = 3$). Data are presented as a relative histamine release (%) to the value of EGCG3''Me (−) and asterisk marks denote significant difference at $^{*}p < 0.01$ (by Student's t-test). (B) Both stimulated cells were lysed and total cellular protein was subjected to Western blot analysis using the anti-phosphorylated MRLC (Thr18/Ser19) antibody and the anti-MRLC antibody.

to impair the degranulation [13,15,22]. To examine whether this MRLC phosphorylation is responsible for the inhibitory effect of EGCG3''Me on degranulation, Western blot analysis for MRLC phosphorylation was performed (Fig. 3B). In the 67LR-shRNA (−) cells, EGCG3''Me clearly reduced the level of phosphorylated MRLC. This lowering effect of EGCG3''Me on the phosphorylation of MRLC was also inhibited by the 67LR knockdown. These findings indicate that the inhibitory effect of EGCG3''Me on degranulation was caused by a modification of myosin cytoskeleton through the binding of EGCG3''Me to the cell-surface 67LR.

RNAi-mediated knockdown of 67LR expression leads to the prevention of EGCG3''Me's ability to suppress the FcεRI expression

To elucidate whether the 67LR is involved in the suppressive effect of EGCG3''Me on FcεRI expression, we examined the effect of EGCG3''Me on the 67LR-downregulated cells. In the 67LR-shRNA (−) cells, the relative FcεRI expression in the cells treated with EGCG3''Me was 62% of the value (MFI: 12.5/20.1) of the non-treated cells (Fig. 4A). However, the 67LR-shRNA (+) cells showed a 88% level (MFI: 17.9/20.4) of FcεRI expression. These results suggest that the suppressive effect of EGCG3''Me is inhibited by the 67LR knockdown. We have recently reported that the inhibition of ERK1/2 phosphorylation was responsible for the suppressive effect elicited by EGCG and its methylated derivatives on FcεRI expression [5,10]. To clarify whether the reductive action of ERK1/2 phosphorylation by EGCG3''Me is transduced through the binding to the 67LR, the 67LR-downregulated cells were treated with EGCG3''Me to examine the level of phosphorylated ERK1/2 (Fig. 4B). Immunoblot analysis showed that EGCG3''Me was able to decrease the phosphorylation of ERK1/2, and its effect was reduced in the 67LR-knockdowned cells. These results indicate that the 67LR is the molecule responsible for transducing the FcεRI-downregulatory signaling elicited by EGCG3''Me.

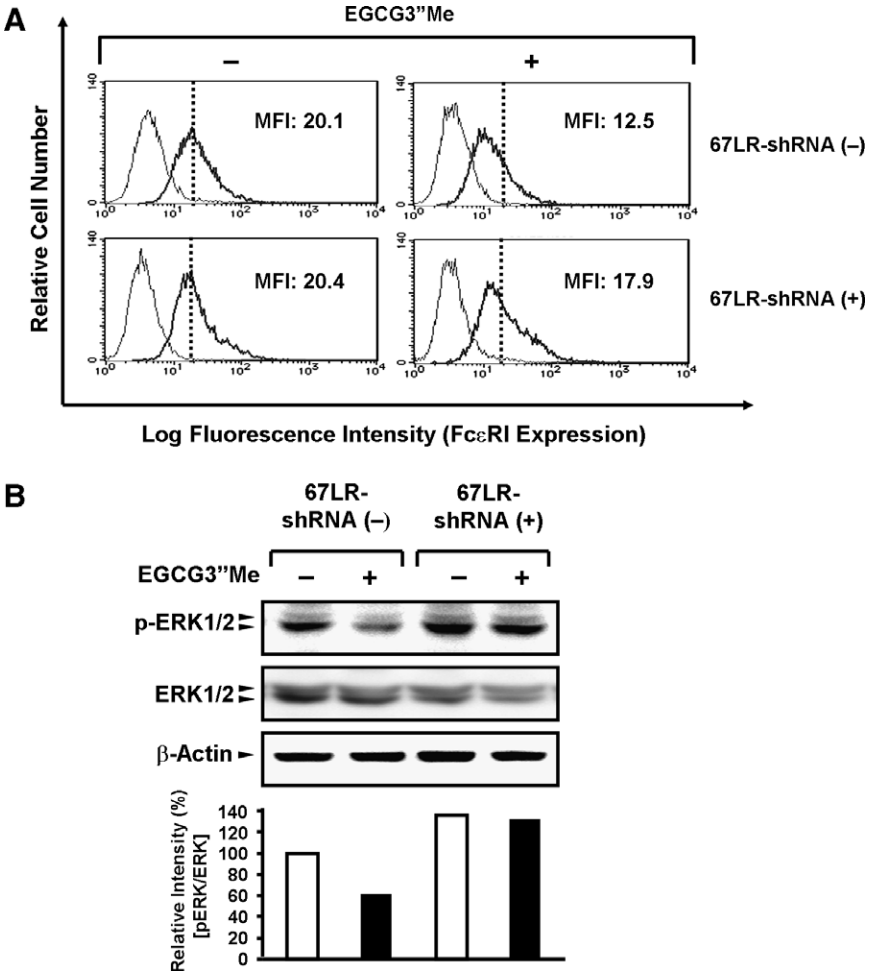

Fig. 4. Effect of the 67LR-downregulation on EGCG3''Me-induced reduction of the FcεRI expression. (A) The 67LR-shRNA (−) or (+) cells were stimulated with EGCG3''Me at 25 μM for 24 h in the presence of catalase plus SOD. Then, the cells were stained with CRA-1, and the fluorescence intensity was determined. The vertical line in figure indicates the peak point for CRA-1 in EGCG3''Me (−) cells. (B) Both cells were treated with EGCG3''Me for 3 h in the presence of catalase plus SOD. ERK1/2 was immunoblotted with anti-phosphorylated ERK1/2 and the anti-ERK1/2 antibody.

Discussion

The 67LR is a nonintegrin cell-surface receptor with high affinity for laminin and plays a key role in tumor invasion and metastasis [23]. Current evidences indicate multifunctional roles of the 67LR in regulations of prion protein propagation and virus infection [24,25]. We recently found that 67LR is the molecular target on the cell surface for EGCG, and that EGCG exerts anti-allergic effects through binding to the 67LR [12,13]. The aim of the present study was broaden the understanding of EGCG3''Me signaling, and a role of 67LR in the EGCG3''Me-induced regulation of FcεRI expression and histamine release was examined in KU812 cells.

The increase of intracellular Ca^{2+} activates degranulation-related cytoskeleton on the basis of actin–myosin interactions [15,16]. We have found that the reduction of MRLC phosphorylation at Thr18/Ser19 caused a modification of actin cytoskeleton [13,21]. This cytoskeletal response associated with a suppressed ability of the cells to release histamine [13]. The present study showed that EGCG3''Me reduced the Thr18/Ser19 phosphorylation of MRLC. The EGCG3''Me's ability to inhibit the MRLC phosphorylation was abolished by knockdown of 67LR. These results suggested that MRLC-regulated cytoskeletal modifications may have an important role in the inhibition of histamine release mediated through the binding of EGCG3''Me to the cell-surface 67LR. This finding is the first report demonstrating the underlying mechanism of degranulation inhibition by EGCG3''Me downstream of the Ca^{2+} influx. Generally, intracellular events of the cell activation leading to histamine release were divided into an interdependent early and late phase. The early phase includes the activation of FcεRI and protein tyrosine kinases, and elevation of intracellular Ca^{2+} levels, and the late phase activation occurs after the influx of Ca^{2+} [14–16]. EGCG3''Me have been shown to inhibit the histamine release from mast cells by blocking the activation of early phase protein tyrosine kinases [9]. We have also reported that EGCG3''Me inhibits the FcεRI crosslinking-induced histamine release by suppression of FcεRI expression [8]. Our present findings that EGCG3''Me-induced downregulation of FcεRI expression is due to the 67LR-mediated inhibition of ERK1/2 phosphorylation provide a novel molecular basis for the suppression of FcεRI expression as well as the regulation of early phase basophil activation. These observations indicate that EGCG3''Me abolishes the histamine release by blocking both early and late phases of basophil or mast cell activation.

Generally, a green tea polyphenol EGCG is known to be unstable under cell culture conditions, and both auto-oxidation and H_2O_2 generation are induced [18,19]. Nevertheless, none of auto-oxidative products of EGCG were observed in the plasma samples from EGCG-administrated mice [26]. These circumstances make it hard to elucidate the intact effect of EGCG itself. Thus, in studies of EGCG or other polyphenolic compounds in cell culture, the addition of catalase and SOD is proposed to stabilize itself and to avoid possible artifacts [18,19]. Our present experiments using such enzymes revealed that anti-allergic effects of EGCG3''Me were exerted through its binding to the cell-surface 67LR. The same result is also obtained in EGCG (data not shown). These observations suggested that the 67LR is able to mediate the ROS-independent extracellular signaling of EGCG and EGCG3''Me.

The inhibitory effects of *O*-methylated EGCGs on mouse type I and IV allergies have been reported to be stronger than those of EGCG [2,3]. EGCG is known to be unstable and degraded easily in animal bodies. In vivo, EGCG is thought to be partly converted to EGCG3''Me [2]. On the other hand, EGCG3''Me is absorbed efficiently and more stable than EGCG in animal and human plasma (unpublished data), suggesting the reason for *O*-methylated EGCGs having potent inhibitory activities to allergies in vivo. Recently, a double-blind clinical trial to treat allergic cedar-polliosis patients with 'Benifuuki' green tea, which is rich in EGCG3''Me, was carried out, and promising results have been obtained [4]. However, the molecular mechanism for biological activities is not fully elucidated. Our present findings that anti-allergic effects of EGCG3''Me were mediated through the cell-surface binding to the 67LR provide a new insight into the molecular basis for biological activities of EGCG3''Me. Future in vivo studies on the relationship between physiological activities of EGCG3''Me and 67LR are required to elucidate a physiological relevant signaling pathway that mediates actions of the *O*-methylated EGCG.

Acknowledgments

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