Cellular Sensing System for Green Tea Polyphenol

Epigallocatechin Gallate

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Abstract
The green tea catechin (−)-epigallocatechin-3-O-gallate (EGCG) is known to exhibit various biological and pharmacological properties. We have identified 67-kDa laminin receptor (67LR) as a cell-surface EGCG receptor that confers EGCG responsiveness to many cancer cells at physiological concentrations. Here we provide an overview of several pathways that sense and manage the activities of EGCG.

EGCG has been shown to rescue mice from lipopolysaccharide (LPS)-induced lethal endotoxemia and downregulate inflammatory responses in macrophages. LPS is one of the most powerful activators of toll-like receptor (TLR)4 signaling and is also well known to induce production of inflammatory mediators. A negative regulator of TLR signaling, Toll-interacting protein (Tollip), is indispensable for mediating the anti-inflammatory action of EGCG, and its protein expression level is upregulated by EGCG through 67LR. Additionally, EGCG can reduce the expression of TLR4 via 67LR.

Using a direct genetic screen, eukaryotic translation elongation factor 1A (eEF1A) is identified as a component responsible for the anti-melanoma activity of EGCG. EGCG induces the dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr-696 and activates myosin phosphatase through both eEF1A and 67LR. Silencing of 67LR, eEF1A, or MYPT1 in tumor cells results in abrogation of EGCG-induced tumor growth inhibition in vivo. Additionally, we found that eEF1A is up-regulated by EGCG through 67LR.

EGCG has been shown to be able to induce apoptotic cell death in multiple myeloma cells through 67LR, while having no significant effect on peripheral blood mononuclear cells (PBMCs). The expression of 67LR was significantly elevated in myeloma cells compared to PBMCs. We found that the apoptosis-inducing activity of EGCG in multiple myeloma cells is attributable to the activation of acid sphingomyelinase (ASM) that hydrolyzes sphingomyelin into ceramide. EGCG induces ASM translocation to the plasma membrane and protein kinase C delta (PKCδ) phosphorylation at Ser664, which was necessary for ASM/ceramide signaling, via 67LR. Furthermore, EGCG activates PKCδ/ASM/ceramide pathway by activating Akt/eNOS/NO/cGMP signaling through 67LR. We also found that the upregulation of cGMP is a rate-determining process of this cell death pathway, and cancer-overexpressed negative regulator of cGMP, PDE5 attenuates the cell death induced by EGCG. Vardenafil, one of the PDE5 selective inhibitors used for treating erectile dysfunction potentiates the anti-cancer effect of EGCG. These results demonstrate that cGMP elevation caused by targeting the overexpressed 67LR and PDE5 in cancer cells may be a useful approach for cancer-specific chemotherapy.
1. Introduction

Green tea is one of the most widely consumed beverages in the world after water. It has been demonstrated that tea constituents exhibit various beneficial health effects such as anti-carcinogenic, anti-oxidative, anti-allergic, anti-virus, anti-hypertensive, anti-atherosclerosis, anti-cardiovascular disease and anti-hypercholesterolemic activities (Bors and Saran 1987; Sano et al. 1995; Sazuka et al. 1995; Cao and Cao 1999; Hodgson et al. 1999; Lambert and Yang 2003a; Kuriyama et al. 2006). Principles for these activities were shown to be a group of polyphenols, catechin. The major green tea catechins are (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epicatechin (Fig. 1). Among the green tea catechins, EGCG is the most abundant, representing ~16.5 wt% of the water extractable fraction of green tea leaves, and most active catechin in various kinds of physiological activities. Because EGCG is not found to a plant except tea, EGCG is regarded as a constituent characterizing green tea.

The bioavailability and biotransformation of tea catechins following tea ingestion has been investigated in human volunteers, and a time to reach maximal concentration in the plasma of 1.5 to 2.5 h after consumption of decaffeinated green tea solids (1.5, 3.0, a and were not detectable by 24 h (Lambert and Yang 2003b). Whereas EGCG and ECG were not detected in the urine, 90% of the urinary EC and EGC were excreted by 8 h. Most of the ingested EGCG apparently does not get into the blood, and absolute EGCG is preferentially excreted through the bile to the colon. Glucuronidation, sulfation, methylation, and ring-fission metabolism represent the major metabolic pathways for green tea catechins. Plasma EC and EGC were present mainly in the conjugated form such as glucuronide and sulfate conjugates, whereas 77% of the EGCG was in the free form. EGCG has also been shown to undergo methylation (Lambert and Yang 2003). Although most of published studies in cell culture systems used 20–100 µM of EGCG, the blood level of EGCG after consuming the equivalent of 2–3 cups of green tea was 0.1–0.6 µM and for an equivalent of 7–9 cups was still lower than 1 µM (Lee et al. 1995). The rather poor bioavailability of tea catechins needs to be considered when we extrapolate results obtained in vitro to situations in vivo.

The polyphenolic structure of tea polyphenols makes them good donors for hydrogen bonding. This hydrogen bonding capacity enables tea polyphenols to bind strongly to proteins and nucleic acids. The difference between in vitro and in vivo systems should be considered in studies attempting to elucidate the mechanisms...
of action of EGCG.

In this review we focused on the current understanding of EGCG sensing mechanisms by which EGCG exerts biological and pharmacological properties.

2. EGCG sensing receptor

Pharmacokinetic studies in humans indicate that the peak plasma concentration after a dose of EGCG is <1.0 µM. It should be noted that most of the effects of EGCG in cell culture systems and cell-free systems are more pronounced at considerably higher concentrations than those observed in the plasma or tissues of animals or in human plasma after administration of green tea or EGCG. Furthermore, the intracellular levels of EGCG are much lower than the concentrations observed in the extracellular levels. Searching for high-affinity proteins that bind to EGCG is the first step to understanding the molecular and biochemical mechanisms of the anti-cancer effects of tea polyphenols. Several proteins that can directly bind with EGCG have been identified with in vitro models (Yang et al. 2009). All of these proteins were demonstrated to be important for the inhibitory activity of EGCG in cell lines, but higher EGCG concentrations than the Kd values were needed. For example, vimentin binds to EGCG with a Kd of 3.3 nM, and functional studies showed that EGCG inhibited the phosphorylation of vimentin at Ser51 and Ser91 by Cdc2 (IC50 = 17 µM) (Ermakova et al. 2005). The difference in effective concentrations is probably due to the nonspecific binding of EGCG to other proteins, which compete with the target proteins. Therefore, it is not clear whether the activities observed with high EGCG concentrations are mediated by specific binding of EGCG to the target proteins.

Next, we investigated the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with 67LR-ablated B16 cells (Umeda et al. 2008a). We confirmed both silencing of 67LR by stable RNAi in B16 cells and attenuation of the inhibitory effect of 1 µM EGCG on cell growth in 67LR-ablated B16 cells in vitro. Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with 67LR-ablated B16 cells, suggesting that 67LR functions as an EGCG receptor not only in vitro but also in vivo. Together, these observations demonstrate that the cell-surface 67LR is the receptor for antitumor action of EGCG at the physiologically relevant concentration. The discovery of EGCG receptor as 67LR has solved some of the discrepancies of the cancer-preventing mechanisms of the anti-cancer effects of tea polyphenols. Furthermore, the intracellular levels of EGCG correlates well with the plasma membrane-associated EGCG level after treating the cells with EGCG (Fujimura et al. 2004).

To investigate whether the 67LR can confer a sensitivity to EGCG at physiologically relevant concentrations, we treated the 67LR-transfected A549 cells with two concentrations of EGCG (0.1 and 1.0 µM); these concentrations are similar to the amount of EGCG found in human plasma after drinking more than two or three cups of tea. The growth of the transfected cells was inhibited at both of these concentrations (Tachibana et al. in vitro 2004). In addition, the growth-suppressive effect was completely eliminated upon treatment with anti-67LR antibody before the addition of EGCG.

We found that all-trans-retinoic acid (ATRA) enhances the binding of EGCG to the cell surface of cancer cells when the binding was monitored on the basis of the increase in response units in a surface plasmon resonance (SPR) assay. To identify candidates through which EGCG inhibits cell growth, we used a subtraction cloning strategy involving cDNA libraries constructed from cells treated or untreated with ATRA. We isolated a single target that allows EGCG to bind to the cell surface. An analysis of the DNA sequence identified this unknown cell surface candidate as the 67-kDa laminin receptor (67LR) (Tachibana et al. 2004). In fact, the expression of this 67LR was enhanced by ATRA treatment.

Human lung cancer A549 cells were used to assess how effectively the 67LR mediates EGCG-mediated growth inhibition. Cells transfected with empty vector and treated with EGCG showed no growth inhibition. However, cells transfected with the gene encoding 67LR and treated with EGCG showed considerable inhibition of tumor growth even at lower concentrations of EGCG. We next tested whether the growth inhibitory activity of EGCG correlates with the binding strength of EGCG to the cell surface. We found increased binding of EGCG to the cell surface of cells transfected with 67LR. EGCG binding to the 67LR-transfected cells was inhibited by treatment with an antibody to 67LR. The predicted Kd value for the binding of EGCG to the 67LR protein is 39.9 nM. Most of the 67LR protein was found to exist in the raft fraction rather than the non-raft fraction (Fujimura et al. 2005), and this distribution pattern correlated well with the plasma membrane-associated EGCG level after treating the cells with EGCG (Fujimura et al. 2004).

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main corresponding to the 161–170 region as the EGCG binding site (Fujimura et al. 2012). Additionally, mass spectrometric analysis revealed that intact (chemically non-altered) EGCG forms the non-covalent complex with 67LR peptide, and there is no ion peak corresponding to the covalent complex of chemically altered EGCG with the 67LR peptide, indicating that EGCG activates 67LR by forming a non-covalent complex with the receptor (Fujimura et al. 2012).

67LR was first discovered by three independent laboratories in 1983 (Rao et al. 1983; Malinoff and Wicha 1983; Jost et al. 1983) through its ability to bind to laminin and be isolated by laminin sepharose. The receptor bound laminin with high affinity with a Kd of 2 nM (Rao et al. 1983). Consequently, this receptor was named 67LR and, more recently, LAMR1 (laminin receptor-1). Its gene, however, was found to encode a protein of only 37-kDa. The discrepancy between these two molecular masses was later resolved by showing that the 37-kDa gene product serves as a monomeric precursor to a 67-kDa dimer (Rao et al. 1989). The exact composition of the 67-kDa dimer and the process by which it is formed remains obscure as evidence supports both a homo (Landowski et al. 1989) and a heterodimer (Butò et al. 1998). 37/67-kDa laminin receptor was shown to be acylated by three fatty acids (palmitate, stearate, and oleate) (Landowski et al. 1998) and fatty acid synthesis is required for 67-kDa laminin receptor formation (Butò et al. 1998). Beyond this not much is known about what regulates the dimerization process.

Expression of the 67LR has been shown to be upregulated in neoplastic cells compared with their normal counterparts and directly correlate with an enhanced invasive and metastatic potential in many malignancies (Sanjuán et al. 1996; Menard et al. 1998). The receptor has been implicated in laminin-induced tumor cell attachment and migration, as well as in tumor angiogenesis, invasion, and metastasis (Mafune et al. 2012). Additionally, mass spectrometric analysis revealed that intact (chemically non-altered) EGCG forms the non-covalent complex with 67LR peptide, and there is no ion peak corresponding to the covalent complex of chemically altered EGCG with the 67LR peptide, indicating that EGCG activates 67LR by forming a non-covalent complex with the receptor (Fujimura et al. 2012).

Phosphorylation of the myosin regulatory light chain (MRLC) at Thr18/Ser19 was shown to regulate the association between myosin II with F-actin. The association of myosin II with F-actin results in the formation of stress fibers in interphase cells and the contractile ring in dividing cells. When human cervical carcinoma HeLa cells were incubated with EGCG, the cells retracted and left intercellular gaps. In addition, disappearance of the stress fibers in the central cell body was observed upon treatment with EGCG and the MRLC phosphorylation was reduced by EGCG treatment. The phosphorylation of MRLC at Thr18/Ser19 has been shown to be necessary for formation of the contractile ring in dividing cells. EGCG treatment significantly increased the percentage of cells in the G2/M phase.

To analyze whether the suppressive effect of EGCG on the MRLC phosphorylation is mediated by the 67LR, RNAi-mediated gene silencing was utilized to knock down the expression of the 67LR (Umeda et al. 2012).
EGCG significantly reduced the phosphorylation of MRLC in the HeLa cells, however in the 67LR-ablated cells, EGCG only slightly reduced the phosphorylation, suggesting that EGCG inhibits the cancer cell growth by reducing the MRLC phosphorylation and this effect is mediated by the 67LR. Epidemiological studies have suggested that the consumption of green tea may decrease colon cancer risk in woman (Yang et al. 2007). We also found that a physiologically achievable concentration of EGCG inhibited cell cycle progression of human colon adenocarcinoma Caco-2 cells through 67LR (Umeda et al. 2008b).

EGCG has been shown to be able to induce growth arrest and subsequent apoptotic cell death in multiple myeloma (MM) cells and primary patient MM cells in vitro, while having no significant effect on growth of normal cells such as peripheral blood mononuclear cells (PBMCs) and fibroblasts (Shammas et al. 2006). Treatment with EGCG (33 mg/kg/d) also led to significant apoptosis in human myeloma cells grown as tumors in SCID mice. The expression of 67LR was significantly elevated in myeloma cell lines and patient samples compared to normal PBMCs. RNAi-mediated inhibition of 67LR expression resulted in abrogation of EGCG-induced apoptosis in myeloma cells, indicating that 67LR plays an important role in mediating EGCG activity in MM while sparing PBMCs. Evaluation of changes in gene expression profile indicates that EGCG treatment activates distinct pathways of growth arrest and apoptosis in MM cells by inducing the expression of death-associated protein kinase 2, the initiation of changes in gene expression profile indicates that EGCG treatment activates distinct pathways of growth arrest and apoptosis in MM cells by inducing the expression of death-associated protein kinase 2, the initiators and mediators of death receptor-dependent apoptosis (Fas ligand, Fas, and caspase 4), p53-like tyrosine kinases and their substrates, generation of the early phase of cell activation of mast cells and basophils (Fujimura et al. 1994; Edgar and Bennett 1997). We found that EGCG inhibited the calcium ionophore A23187-induced histamine release from the human basophilic KU812 cells and could not inhibit the increase of the intracellular Ca²⁺ level after stimulation with A23187 (Fujimura et al. 2006). After treatment of KU812 cells with the anti-67LR antibody, cells were incubated with diacylglycerol, and elevation of intracellular Ca²⁺ levels (Turner and Kinet 1999; Rivera 2002). The late phase of the activation, which occurs after the influx of Ca²⁺, includes the fusion of secretory granules with the membrane and dramatic morphological changes due to remodeling of actin cytoskeleton, which undergo extensive membrane ruffling (Pfeiffer et al. 1985; Choi et al. 1994, Edgar and Bennett 1997). We found that EGCG inhibited the calcium ionophore A23187-induced histamine release from the human basophilic KU812 cells and could not inhibit the increase of the intracellular Ca²⁺ level after stimulation with A23187 (Fujimura et al. 2006). This result suggested that the effect of EGCG on histamine release occurs after the elevation of the intracellular Ca²⁺ concentration. Thr18/Ser19 phosphorylation of MRLC has been reported to be temporally correlated with degranulation in the rat basophilic RBL-2H3 cells, and the inhibition of MRLC phosphorylation has been shown to impair the degranulation (Ludowyke et al. 1989). Although EGC, having no ability to inhibit histamine release, showed no inhibitory effect on MRLC phosphorylation, EGCG clearly reduced the level of phosphorylated MRLC (Fujimura et al. 2006). After treatment of KU812 cells with the anti-67LR antibody, cells were incubated with EGCG, and further challenged with A23187. The reductive effect of EGCG on the histamine release was almost completely inhibited in cells treated with the anti-67LR antibody (Fujimura et al. 2006). An experiment using such 67LR-downregulated cells revealed a significant abrogation of the inhibitory effect of EGCG on degranulation. Furthermore, the lowering effect of

4. Anti-allergic actions of EGCG through EGCG sensing receptor 67LR

Mast cells and basophils play a central role in immediate allergic reactions mediated by IgE. Binding of multivalent allergens to specific IgE attached to the FceRI on the surface of mast cells or basophils leads to the release of both preformed and newly generated inflammatory mediators such as histamine. These mediators ultimately cause various symptoms including atopic dermatitis, bronchial asthma, and food allergy (Ravetch and Kinet 1991; Metzger 1992). The early phase of cell activation of mast cells and basophils includes the phosphorylation and activation of protein tyrosine kinases and their substrates, generation of the second messengers such as inositol trisphosphate and diacylglycerol, and elevation of intracellular Ca²⁺ levels (Turner and Kinet 1999; Rivera 2002). The late phase of the activation, which occurs after the influx of Ca²⁺, includes the fusion of secretory granules with the membrane and dramatic morphological changes due to remodeling of actin cytoskeleton, which undergo extensive membrane ruffling (Pfeiffer et al. 1985; Choi et al. 1994, Edgar and Bennett 1997). We found that EGCG inhibited the calcium ionophore A23187-induced histamine release from the human basophilic KU812 cells and could not inhibit the increase of the intracellular Ca²⁺ level after stimulation with A23187 (Fujimura et al. 2006). This result suggested that the effect of EGCG on histamine release occurs after the elevation of the intracellular Ca²⁺ concentration. Thr18/Ser19 phosphorylation of MRLC has been reported to be temporally correlated with degranulation in the rat basophilic RBL-2H3 cells, and the inhibition of MRLC phosphorylation has been shown to impair the degranulation (Ludowyke et al. 1989). Although EGC, having no ability to inhibit histamine release, showed no inhibitory effect on MRLC phosphorylation, EGCG clearly reduced the level of phosphorylated MRLC (Fujimura et al. 2006). After treatment of KU812 cells with the anti-67LR antibody, cells were incubated with EGCG, and further challenged with A23187. The reductive effect of EGCG on the histamine release was almost completely inhibited in cells treated with the anti-67LR antibody (Fujimura et al. 2006). An experiment using such 67LR-downregulated cells revealed a significant abrogation of the inhibitory effect of EGCG on degranulation. Furthermore, the lowering effect of

doi:10.5047/agbm.2014.00402.0019 © 2014 TERRAPUB, Tokyo. All rights reserved.
EGCG on the phosphorylation of MRLC was also inhibited either by treatment with the anti-67LR antibody or 67LR-knockdown. These findings indicate that the inhibitory effect of EGCG on degranulation was caused by a modification of myosin cytoskeleton through the binding of EGCG to 67LR on the cell surface. When the basophilic cells were stimulated with A23187 in the presence of EGCG, membrane ruffling was inhibited and a biased F-actin accumulation was observed. Furthermore, this EGCG-induced actin remodeling was abolished in both anti-67LR antibody-treated cells and 67LR-knockdowned cells (Fujimura et al. 2006). Our findings indicated that EGCG-induced actin remodeling is caused by lowering MRLC phosphorylation mediated through the binding of EGCG to the 67LR. Thus, these cytoskeletal modifications may have an important role in the inhibition of histamine release by EGCG.

FcεRI plays a central role in the induction and maintenance of IgE-mediated allergic responses such as atopic dermatitis, bronchial asthma, and food allergy. Analysis of FcεRIα-deficient mice demonstrated that IgE was unable to bind to the cell surface of mast cells, thereby inhibiting the induction of degranulation through IgE binding (Dombrowicz et al. 1993). 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 found that EGCG was able to decrease the cell-surface expression of FcεRI in human basophilic KU812 cells. Total cellular expression of the FcεRIα chain decreased upon treatment with EGCG. We also found that EGCG has an ability to inhibit the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) (Fujimura et al. 2004). This inhibition was involved in downregulation of FcεRI expression by EGCG. Moreover, the inhibitory effect elicited by EGCG on ERK1/2 was prevented by disruption of lipid rafts. Accordingly, 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. We also demonstrated that the suppressive effect of EGCG was inhibited by the knockdown of 67LR.

**Fig. 2.** EGCG sensing pathway for the anti-allergic and anti-inflammatory actions through 67-kDa laminin receptor (67LR). The suppression of MRLC phosphorylation through the cell-surface binding to the 67LR contributes to the inhibitory effect of EGCG on the histamine release from basophils. The 67LR also mediates the EGCG-induced suppression of FcεRI expression in basophils by reducing ERK1/2 phosphorylation. 67LR and Tollip are indispensable for mediating anti-inflammatory action of EGCG on TLRs signaling induced by LPS and PGN.
ability of EGCG to decrease the phosphorylation of ERK1/2 was reduced in the 67LR-knocked down cells. These results indicate that the effect of EGCG on ERK1/2 phosphorylation correlates with the expression of 67LR, which implies that the 67LR is the molecule responsible for transducing the EGCG’s downregulatory signaling of the FcεRI (Fig. 2).

The O-methylated derivatives of EGCG, (−)-epigallocatechin-3-O-(3-O-methyl)-gallate (EGCG3′Me) (Fig. 1) and (−)-epigallocatechin-3-O-(4-O-methyl)-gallate (EGCG4′Me), which were isolated from tea leaves, have been shown to inhibit allergic reactions in vitro (Sano et al. 1999; Tachibana et al. 2000). The inhibitory effects of O-methylated EGCG on mouse type I and IV allergies in vivo were more potent than that of EGCG (Sano et al. 1999; Suzuki et al. 2000). These catechins also strongly inhibited mast cell activation between the prevention of tyrosine phosphorylation of cellular protein, histamine/leukotriene release, and interleukin-2 secretion after FcεRI cross-linking (Maeda-Yamamoto et al. 2004). A double-blind clinical trial to treat allergic celiac pollinosis patients with ‘Benifuuki’ green tea rich in EGCG3′Me was carried out, and promising results have been obtained by using a protocol of drinking of 1.5 g of tea powder with water twice a day for 13 weeks (Maeda-Yamamoto et al. 2007). We have found that EGCG3′Me can inhibit histamine release and suppress the FcεRI expression in human basophilic KU812 cells that the same as EGCG (Tachibana et al. 2000; Fujimura et al. 2002). RNAi-mediated knockdown of 67LR expression resulted in a decreased activity of EGCG3′Me in macrophages (Byun et al. 2010). We also reported that 67LR and Tollip are indispensable for mediating anti-inflammatory action of EGCG on LPS-induced activation of downstream signaling pathways and target gene expressions in murine macrophages. Additionally, we found that EGCG reduced the TLR4 expression through 67LR. Interestingly, EGCG induced a rapid upregulation of Tollip protein, a negative regulator of TLR-signaling, and this EGCG action was prevented by 67LR silencing or anti-67LR antibody treatment. RNAi-mediated silencing of Tollip impaired the TLR4 signaling inhibitory activity of EGCG. Taken together, these findings demonstrate that 67LR plays a critical role in mediating anti-inflammatory action of a physiologically relevant EGCG and Tollip expression could be modulated through 67LR in macrophages (Byun et al. 2010) (Fig. 2).

Peptidoglycan (PGN), a major component of the cell wall of Gram-positive bacteria, is one of the most powerful activators of TLR2 signaling (Fourrier and Philpott 2005; Uckay et al. 2007). PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever and septic shock. We also reported that 67LR and Tollip are indispensable for mediating anti-inflammatory action of EGCG on TLR2 signaling (Byun et al. 2011).

6. EGCG sensing-relating molecules

As previously shown, 67LR mediates anti-cancer action of EGCG as the cell surface receptor. Therefore, we tried to illuminate the cell signaling pathway

5. Anti-inflammatory actions of EGCG through EGCG sensing receptor 67LR

Toll-like receptors (TLRs) are important in the activation of the innate immune response and are pathogen recognition proteins that have important roles in detecting microbes and initiating inflammatory responses (Takeda and Akira 2005). Recognition of microbial components by TLRs plays a central role in the immune system’s decision to respond or not to a particular microbial infection. Lipopolysaccharide (LPS), a major component of the outer cell wall of Gram-negative bacteria, is one of the most powerful activators of TLR4 signaling and is also well known to induce production of inflammatory mediators, such as TNF-α, IL-6, and NO, and activation of the MAPK signaling pathway and NF-κB, leading to death from endotoxic shock in animal models (Takeuchi and Akira 2001; Cohen 2002; Fujihara et al. 2003). EGCG has been shown to rescue mice from LPS-induced lethal endotoxemia and downregulate inflammatory responses in macrophages (Li et al. 2007).

Recently, we found the molecular basis for the downregulation of TLR4 signal transduction by EGCG at 1 μM in macrophages (Byun et al. 2010). Anti-67LR antibody treatment or RNAi-mediated silencing of 67LR resulted in abrogation of the inhibitory action of EGCG on LPS-induced activation of downstream signaling pathways and target gene expressions in murine macrophages. Additionally, we found that EGCG reduced the TLR4 expression through 67LR. Interestingly, EGCG induced a rapid upregulation of Tollip protein, a negative regulator of TLR-signaling, and this EGCG action was prevented by 67LR silencing or anti-67LR antibody treatment. RNAi-mediated silencing of Tollip impaired the TLR4 signaling inhibitory activity of EGCG. Taken together, these findings demonstrate that 67LR plays a critical role in mediating anti-inflammatory action of a physiologically relevant EGCG and Tollip expression could be modulated through 67LR in macrophages (Byun et al. 2010) (Fig. 2).
mediated after the binding of EGCG to 67LR and its biological and physiological significance for the cancer-preventive activity of EGCG \textit{in vivo}.

6-1. eEF1A

In an attempt to elucidate the pathways involved in the anticancer action of EGCG, we applied genetic suppressor element (GSE) methodology (Umeda et al. 2008a). GSEs are short cDNA fragments encoding peptides acting as dominant inhibitors of protein function or antisense RNAs inhibiting gene expression. GSEs behave as dominant selectable markers for the phenotype associated with the repression of the gene from which they derived, thus allowing identification of this gene. For identifying genes mediating cell sensitivity to EGCG, we selected GSEs conferring resistance to EGCG. To search for the mediators of EGCG-induced cell growth inhibition in B16 mouse melanoma cells, we utilized a targeted genetic screen with a GSE complementary DNA library. Among genetic elements protecting cells from EGCG-induced cell growth inhibition, we isolated a GSE that encoded the N terminus of eukaryotic translation elongation factor 1A (eEF1A). eEF1A is an important component of the eukaryotic translation apparatus and is also known as a multifunctional protein that is involved in a large number of cellular processes (Negrutskii and El’skaya 1998).

To investigate the role of eEF1A in EGCG-induced cell growth inhibition, we used stable RNAi to silence eEF1A expression in B16 cells (Umeda et al. 2008a). Remarkably, silencing of eEF1A attenuated the inhibitory effect of 1 \mu M EGCG on cell growth. In contrast, overexpression of eEF1A enhanced the inhibitory effects of 1 \mu M EGCG on cell growth. This concentration is similar to the amount of EGCG found in human plasma after drinking more than two or three cups of green tea. Given this, we investigated the effect of EGCG on the phosphorylation of MYPT1 at Thr-696 and Thr-853. Intriguingly, although the phosphorylation level at Thr-853 was unaffected by EGCG, EGCG induced the dephosphorylation of MYPT1 at Thr-696. Further, this effect correlated with EGCG-induced reduction of the MRLC phosphorylation, suggesting that EGCG activates myosin phosphatase by reducing the MYPT1 phosphorylation level at Thr-696. Next, we investigated whether MYPT1 is involved in anticancer action of EGCG \textit{in vivo} (Umeda et al. 2008a). In B16 cells, physiological concentrations of EGCG reduced the MYPT1 phosphorylation at Thr-696 and the MRLC phosphorylation. We confirmed both the silencing of MYPT1 by stable RNAi in B16 cells and the attenuation of the inhibitory effect of 1 \mu M EGCG on cell growth in MYPT1-ablated B16 cells in vitro. We tested the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with MYPT1-ablated B16 cells (Umeda et al. 2008a). Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with eEF1A-ablated B16 cells, indicating that eEF1A is involved in EGCG-induced cancer prevention. These results support our conclusion that eEF1A serves as a mediator for EGCG-induced cancer prevention.

6-2. MYPT1

As described previously, EGCG-induced cell growth inhibition may result from the reduction of the phosphorylation of MRLC at Thr-18/Ser-19 through 67LR (Umeda et al. 2005). The activity of myosin phosphatase is known to be inhibited by phosphorylation of its targeting subunit MYPT1 at Thr-696 and Thr-853. We tested the effect of EGCG on the phosphorylation of MYPT1 at Thr-696 and Thr-853. Intriguingly, although the phosphorylation level at Thr-853 was unaffected by EGCG, EGCG induced the dephosphorylation of MYPT1 at Thr-696. Further, this effect correlated with EGCG-induced reduction of the MRLC phosphorylation, suggesting that EGCG activates myosin phosphatase by reducing the MYPT1 phosphorylation level at Thr-696. Next, we investigated whether MYPT1 is involved in anticancer action of EGCG \textit{in vivo} (Umeda et al. 2008a). In B16 cells, physiological concentrations of EGCG reduced the MYPT1 phosphorylation at Thr-696 and the MRLC phosphorylation. We confirmed both the silencing of MYPT1 by stable RNAi in B16 cells and the attenuation of the inhibitory effect of 1 \mu M EGCG on cell growth in MYPT1-ablated B16 cells in vitro. We tested the effect of oral administration of EGCG on subcutaneous tumor growth in C57BL/6N mice challenged with MYPT1-ablated B16 cells (Umeda et al. 2008a). Tumor growth was significantly retarded in EGCG-administered mice implanted with the B16 cells harboring a control shRNA, whereas tumor growth was not affected by EGCG in the mice implanted with MYPT1-ablated B16 cells, suggesting that MYPT1 is indispensable for EGCG-induced cancer prevention.

In both 67LR-ablated HeLa cells and eEF1A-ablated HeLa cells, the inhibitory effect of EGCG on both the phosphorylation of MYPT1 at Thr-696 and the phospho-
Phosphorylation of MRLC was attenuated. In addition, EGCG-induced actin cytoskeleton rearrangement was no longer observed in MYPT1-, eEF1A-, or 67LR-ablated HeLa cells. The involvement of MYPT1 in downstream EGCG-triggered signaling from both 67LR and eEF1A was further documented by confirming abrogation of 1 μM EGCG-induced reduction of the MYPT1 phosphorylation level at Thr-696 and the MRLC phosphorylation in 67LR- or eEF1A-ablated B16 cells. These results suggest that MYPT1 is involved in downstream EGCG signaling from both 67LR and eEF1A (Edmonds et al. 1996). Characterizing the mechanisms by which EGCG induces reduction of the MYPT1 phosphorylation at Thr-696 and reorganization of actin cytoskeleton through eEF1A should help in a more precise understanding of cytoskeleton organization.

**6-3. Protein kinase Cδ (PKCδ)/acid sphingomyelinase (ASM)**

EGCG induces apoptosis in several cancers, and the EGCG-induced cell killing requires 67LR expression in human AML (Britschgi et al. 2010) and MM patient cells (Shammas et al. 2006) as described. However, the downstream mechanisms are still unclear.

EGCG inhibits cell proliferation of primary myeloma and U266 cells, as well as cervical carcinoma HeLa cells, but does not affect PBMCs (Tsukamoto et al. 2012). EGCG also reduced the survival of U266 and primary MM cells, but did not affect the viability of HeLa cells. We have shown that dephosphorylation of MYPT1 at Thr696 mediates the EGCG-induced growth inhibition of B16 melanoma cells and HeLa cells (Umeda et al. 2008a). In immunoblotting, EGCG dose-dependently reduced phosphorylation of MYPT1 at Thr696 in HeLa cells, but did not affect phosphorylation in MM cells. Taken together, these results suggest that EGCG induces apoptotic cell death through 67LR, but not via the MYPT1 pathway, in MM cells.

Increases in membrane fluidity and clustering of lipid rafts play crucial roles in apoptosis (Chen et al. 1996). To examine the effect of EGCG on lipid raft clustering, we evaluated FRET signaling after staining with CTX-Alexa Fluor® 488 and CTX-Alexa Fluor® 594 before stimulation with EGCG. EGCG dose- and time-dependently increased lipid raft clustering, but treatment with EC, which lacks biological activity, did not induce lipid-raft clustering in U266 cells. We examined whether EGCG induced lipid clustering was mediated through 67LR. To block the interaction of EGCG and 67LR, U266 cells were treated with either an anti-67LR antibody or control antibody.

Pretreatment with the anti-67LR antibody could block EGCG-induced lipid raft clustering, whereas pretreatment with the control antibody did not. We next investigated the effect of cholesterol, a membrane-stabilizing agent, on EGCG-induced apoptosis. Exposure of myeloma cells to cholesterol inhibited lipid-raft clustering and apoptosis, suggesting that the apoptotic activity of EGCG is caused by lipid-raft clustering.

Lipid-raft clustering occurs after generation of ceramide by acid sphingomyelinase (ASM) (Lacour et al. 2004; Rebillard et al. 2007). ASM acts on membrane sphingomyelin to generate ceramide, which mediates cell death induced by diverse stimuli, such as ionizing radiation, chemotherapeutic agents and UV-light. The expression of ASM was abnormally elevated in all myeloma cell lines relative to normal PBMCs. EGCG dose-dependently activated ASM in U266 cells, human MM cell lines and primary MM cells, but did not affect normal PBMCs. Moreover, pretreatment with an anti-67LR antibody blocked EGCG-induced activation of ASM, suggesting that 67LR mediates ASM activation by EGCG. ASM hydrolyses cell-surface sphingomyelin after directly translocating to the intracellular membrane during cellular stress responses such as ligation of death receptors (CD95), cisplatin or UV radiation (Lacour et al. 2004; Molinolo and Gajate 2006). EGCG increased ASM in the cell-membrane fraction, and pretreatment with the anti-67LR antibody blocked this translocation (Tsukamoto et al. 2012). Moreover, an ASM-specific inhibitor, desipramine, blocked EGCG-induced ceramide production. Taken together, these observations show that EGCG modulates the sphingolipid pathway through activating ASM via 67LR.

Desipramine, an ASM inhibitor, blocked EGCG-induced cell death in U266, MPC-11 and primary MM cells, indicating that ASM activity mediated this activity. Desipramine also blocked EGCG-induced apoptotic cell death, as well as normalizing EGCG-induced lipid-raft clustering in U266 cells. Transfection of U266 cells with an shRNA expression vector to reduce ASM expression abolished EGCG-induced ASM activation and apoptosis. Collectively, these results suggest that ASM is necessary for EGCG-induced lipid-raft clustering, leading to apoptotic cell death in MM cells.

Protein kinase Cδ (PKCδ) is critical for the induction of apoptosis (Frasch et al. 2000; Matassa et al. 2001; Anantharam et al. 2002). Therefore we tested whether PKCδ was involved in EGCG induced activation of the ASM/ceramide pathway. EGCG dose-dependently enhanced generic PKC activity. EGCG treatment of U266 cells for 5 min led to phosphorylation at Ser664 that was dose-dependent, but did not affect phosphorylation of Tyr155 and Thr507. More-
over, EGCG increased Ser664 phosphorylation in three MM cell lines and primary MM cells, but not in normal PBMCs.

Next, we examined whether EGCG induced activation of PKCδ was mediated through 67LR. To block the interaction of EGCG and 67LR, U266 cells were treated with either an anti-67LR antibody or control antibody. EGCG-induced PKCδ phosphorylation at Ser664 was not observed in cells pretreated with the anti-67LR antibody, suggesting that 67LR mediates EGCG-induced phosphorylation of PKCδ at Ser664. Furthermore, treatment with the PKCδ-specific inhibitor rottlerin abolished the EGCG-induced activation of ASM. Silencing of ASM in MM cells did not affect EGCG-induced PKCδ phosphorylation at Ser664. Overall, these results suggest that EGCG-induced ASM activation is a secondary event that occurs after activation of PKCδ.

To evaluate the involvement of the PKCδ/ASM pathway in the killing activity of EGCG on MM cells in vivo, we examined the effect of EGCG on activation of caspase 3, PKCδ and ASM in tumor cells. ARH-77 cells were injected subcutaneously into SCID mice. Oral administration of EGCG promoted the cleavage of caspase 3, a key mediator of apoptosis, in tumor cells. Moreover, EGCG induced PKCδ phosphorylation at Ser664 in tumors, indicating the in vivo anti-myeloma activity of EGCG. We also tested the effects of oral or intraperitoneal EGCG in an MPC-11 tumour xenograft model. The intraperitoneal injection of EGCG increased levels of cleaved caspase 3 in tumor cells, as well as PKCδ phosphorylation at Ser664 and ASM activation. Orally administered EGCG produced similar effects on caspase 3, PKCδ and ASM activation (Tsukamoto et al. 2012). Consistent with the in vitro results, these results demonstrate that EGCG activates PKCδ and ASM in MM cells in vivo.

6-4. NO/cGMP

Activation of the PKCδ/ASM pathway is involved in downstream effectors in EGCG-induced apoptosis (Tsukamoto et al. 2012). Next, we investigated the mechanisms by which cancer-overexpressed 67LR activates PKCδ/ASM pathway as a novel death receptor. 67LR has been shown to be involved in shear stress-

Fig. 4. EGCG sensing pathway for inducing cancer cell death through 67LR. 67LR activates the peculiar apoptotic signaling Akt/eNOS/NO/sGC/cGMP/PKCδ/ASM pathway. Furthermore, upregulation of cGMP could be a rate-determining process of 67LR-dependent cell death. The combination of EGCG as a cancer-specific cGMP inducer with an inhibitor targeting cancer-overexpressed PDE5 could be a useful strategy for cancer-selective chemotherapy. Reprinted with permission from ILSI, 116, Tachibana, Green tea catechin sensing system, 6–13, Fig. 4, © 2014, ILSI Japan.
induced eNOS expression in normal endothelial cells (Gloe et al. 1999). Therefore, we investigated the role of NO in 67LR-dependent cell death (Kumazoe et al. 2013a). Primary MM cells derived from a MM patient, MM cell lines U266 and RPMI8226, and normal PBMCs were treated with EGCG for 3 hours, and NO production was evaluated. EGCG induced NO production in MM cell lines and primary MM cells, but had no effect on PBMCs from healthy donors. We also found that EGCG elicited eNOS phosphorylation at Ser1177, which is involved in eNOS activation (Kumazoe et al. 2013a). Akt inhibitor attenuated EGCG-induced phosphorylation of eNOS at Ser1177. Taken together, our findings indicate that EGCG induces NO production through 67LR-dependent activation of Akt and eNOS.

Next, we investigated the effect of EGCG on intracellular cGMP, a crucial mediator in NO-induced signaling (Arnold et al. 1977). EGCG elevated the amount of cGMP in a dose-dependent manner in primary MM cells and U266 cells, but had no effect on normal PBMC. To investigate whether cGMP activates a 67LR-dependent apoptotic pathway, we examined the effects of the cell-permeable cGMP analog dibutyryl-cGMP on the cGMP level. To our knowledge, cGMP-induced ASM activation in a dose-dependent manner. Pretreatment of U266 cells with anti-67LR antibody inhibited EGCG-induced cGMP elevation. NO increased the intercellular cGMP level by activating soluble guanylate cyclase (sGC). The sGC inhibitor NS-2028 prevented the cGMP upregulation induced by EGCG. Furthermore, NS-2028 pretreatment also attenuated EGCG-induced cell death and ASM activation. Taken together, these results suggested that the 67LR/Akt/eNOS/sGC/cGMP pathway mediates EGCG-induced cell death (Fig. 4). Other tea catechins and their structurally related compounds have little affinity for 67LR (Tachibana et al. 2004) and did not affect the intracellular cGMP level. To our knowledge, this is the first study to demonstrate that cGMP initiates an apoptotic pathway by activating ASM.

6-5. PDEs

cGMP has a crucial role in EGCG-induced MM-specific cell death. EGCG inhibited U266 cell growth with an IC50 of 23.2 µM. This concentration was much higher than the plasma concentration previously observed in clinical trials (Shanafelt et al. 2009). EGCG at physiologically achievable levels could induce NO production but could not upregulate the level of cGMP sufficiently to induce MM cell death. These results suggested that upregulation of cGMP may be a “choke point” of the EGCG-induced apoptotic signaling pathway. PDEs are enzymes that inactivate cGMP signaling by hydrolyzing the 3′,5′-phosphodiester bond. We hypothesized that the PDEs may protect MM cells from EGCG-induced cell death by downregulating the cGMP level. To determine the effect of various PDEs on the anti-MM effect of EGCG, myeloid cell lines were pretreated with inhibitors of different PDEs (Kumazoe et al. 2013a). Significant inhibition of cell proliferation was observed when EGCG was combined with the PDE5-selective inhibitors zaprinast, methoxyquinazoline (MQZ), sildenafil, and vardenafil. PDE5 is one of the major negative regulators of cGMP signaling. However, the expression of PDE5 in MM cells is not known. The protein levels of PDE5 and 67LR increased substantially in the MM cells of 10 patients as well as all human MM cell lines compared with those in normal PBMCs of 10 healthy donors. Surprisingly, a significant correlation was observed between expression of 67LR and PDE5. These data may provide a rational explanation for the insensitivity of MM cells to low concentrations of EGCG despite the high expression of 67LR. To confirm the role of PDE5 in EGCG resistance, we investigated the effect of PDE5 silencing. Reduction in the PDE5 protein expression markedly potentiated the anti-MM effect of EGCG. Furthermore, the PDE5 inhibitor vardenafil, which is used for treating erectile dysfunction (Porst et al. 2001), had no effect on the number of viable normal PBMCs from healthy donors, but significantly enhanced the killing activity of EGCG on primary MM cells from patients and from the MM cell lines U266, RPMI8226, and ARH-77. Treatment with EGCG and vardenafil combined resulted in greater inhibition of the growth of U266 cells, with an IC50 of 1.4 µM compared with 23.2 µM for EGCG alone. Isobologram analyses showed that the growth-inhibitory effects of combined treatment with EGCG and vardenafil on the growth of U266 cells and RPMI8226 cells were synergetic. We also found that vardenafil sensitized U266 cells to EGCG3°Me. Conversely, vardenafil in combination with the other catechins did not induce cell death in MM cells.

To evaluate the in vivo activity of EGCG and vardenafil in combination, female BALB/c mice were inoculated subcutaneously (Kumazoe et al. 2013a). Af-
The appearance of palpable tumors, mice were then given a single i.p. injection of 15 mg/kg EGCG and/or 5 mg/kg vardenafil dissolved in physiological saline (0.9%). After 6 hours, tumors were excised to evaluate the effect on the activity of PKCδ and ASM. Injection i.p. of EGCG and vardenafil in combination increased PKCδ phosphorylation at Ser662 (corresponding to human p-PKCδ Ser664) as well as ASM activity. Moreover, this combination significantly upregulated the cleavage of caspase-3, a key mediator of apoptosis in tumor cells. Collectively, these results suggested that vardenafil inhibited the anti-MM effect of EGCG through amplification of the downstream effectors PKCδ and ASM. To evaluate the long-term effect of EGCG and vardenafil combination on tumor growth, female BALB/c mice were given i.p. injection of 15 mg/kg EGCG and/or 5 mg/kg vardenafil every 2 days. The combination of EGCG and vardenafil significantly suppressed tumor growth in the mice. Furthermore, log-rank analyses of the Kaplan-Meier survival curves showed a significant increase in the survival of mice treated with EGCG and vardenafil in combination compared with mice treated with saline, EGCG alone, or vardenafil alone. To confirm whether PDE5 protects MM cells from EGCG-induced cell death in vivo, BALB/c mice were inoculated subcutaneously with control shRNA or the PDE5 shRNA expression vector; after the appearance of palpable tumors, mice were given a single i.p. injection of 15 mg/kg EGCG every 2 days. A reduction in the expression of PDE5 protein markedly potentiated the anti-MM effect of EGCG in vivo. To determine whether the combination of EGCG and PDE5 inhibitor induced apoptosis, we undertook antibody-blocking experiments, which demonstrated that 67LR mediated the apoptosis-inducing effect of this combination (Kumazoe et al. 2013a). We also found that vardenafil potentiated EGCG-induced upregulation of cGMP in MM cells and enhanced the anti-MM effect of the sGC activator BAY 41-2272. To confirm the role of ASM in the anticancer effect of EGCG and vardenafil combined, we transfected U266 myeloma cells with a lentivirus encoding scrambled control shRNA or shRNA against ASM. Remarkably, silencing of ASM abrogated the inhibitory effect of the combination on U266 cells. Collectively, these results demonstrated that PDE5 inhibitor potentiated EGCG-induced apoptosis by enhancing the 67LR/cGMP/ASM-dependent signaling pathway in MM cells (Fig. 4).

Next, we investigated the effect of EGCG and vardenafil in various types of cancer (Kumazoe et al. 2013b). The combination of EGCG and vardenafil inhibited the proliferation of the gastric cancer cell line MKN45, the pancreatic cancer cell line PANC-1, the prostate cancer cell line PC3, and acute myeloid leukemia cells but did not affect normal human diploid fibroblasts (NHDFs) or normal HUVECs. Immunohistochemical analyses of paraffin-embedded tissue sections showed that levels of 67LR and PDE5 were elevated in various types of human cancers (gastric, pancreatic, prostate, and breast) compared with their normal counterparts. The combination of EGCG and vardenafil inhibited proliferation of the human breast cancer cell line MDA-MB-231-RFP in vitro. To evaluate the in vivo activity of EGCG and vardenafil in combination on MDA-MB-231 cells, the cells were injected subcutaneously into female nude mice; after the appearance of palpable tumors, mice were given i.p. injections of 15 mg/kg EGCG and/or 5 mg/kg vardenafil every 2 days. EGCG and vardenafil in combination significantly suppressed tumor growth, and did not increase serum levels of ALT/AST. In addition, the combination induced apoptosis in the pancreatic cancer cell line PANC-1. A prior study showed that a PANC-1 cell subpopulation propagates colony formation and has the properties of stem cells (Gou et al. 2007). The combination of EGCG and vardenafil treatment inhibited colony formation in PANC-1 cells.

7. Factors that modulate the EGCG sensing receptor 67LR

7.1. Vitamin A

As previously shown, the concentrations of EGCG that are required to elicit the anticancer effects in a variety of cancer cell types are much higher than the peak plasma concentration that occurs after drinking an equivalent of 10 cups of green tea. To obtain the anticancer effects of EGCG, mice were given a reasonable concentration in daily life, we investigated the combination effect of EGCG and food ingredient that may enhance the anticancer activity of EGCG. Vitamin A, also known as retinol, participates in physiological activities related to the immune system, maintenance of epithelial and mucous tissues, growth, reproduction, and bone development. It comes from animal sources, such as eggs, meat, milk, cheese, cream, liver, kidney, cod and halibut fish oil. In vitro and in animal models, it has been demonstrated that vitamin A is involved in the regulation and promotion of growth and differentiation of many cells (Ozer et al. 2005). All-trans-retinoic acid (ATRA), the active derivative of vitamin A, has been well documented as a growth and differentiation factor in many tissues and cells, and proved to be an effective treatment to many diseases including cancers (Xia et al. 2006; Haque et al. 2007). Retinoids exert their physiological activities through retinoid receptor nuclear proteins that belong to the superfamily of steroid/thyroid hormone receptors, of which there are two classes, retinoic acid receptors (RARs) and the retinoic-X receptors (RXRs), each of which has three subtypes, a, b, and c. The natu-
ral ligands for the RARs are ATRA and its stereoisomers 9-cis-RA and 13-cis-RA.

We previously showed that ATRA enhances the expression of 67LR on MCF-7 cells (Tachibana et al. 2004). To determine whether ATRA enhances anti-tumor effect of EGCG in vivo, we examined the 67LR expression on B16 melanoma cells by using Western blot analysis after treatment with different concentrations of ATRA (Lee et al. 2010). ATRA enhanced the expression of 67LR in a dose dependent manner. We also found that ATRA treatment increased the cell surface expression of the 67LR as compared with the expression in the control cells. We next examined the effects of combined EGCG and ATRA treatment on cell growth of B16 cells. Combination treatment with ATRA (0.1 µM) and EGCG at a physiological concentration (0.5 µM) significantly suppressed the number of B16 cells to 52.4% of the control, whereas treatment with EGCG or ATRA alone did not inhibit cell growth. These results suggest that ATRA enhances EGCG-induced cell growth inhibition through 67LR upregulation in B16 cells. Cell surface binding of EGCG was assessed using SPR biosensor assay. We found that ATRA significantly enhances the binding of EGCG to the surface of B16 cells. To investigate if the participation of 67LR in ATRA-induced the cell growth inhibitory activity of EGCG, B16 cells were treated with an anti-67LR antibody. The growth of the cells treated with a control antibody was inhibited by the combined EGCG and ATRA treatment. This growth-suppressive effect was eliminated upon treatment with an anti-67LR antibody. Together, these observations show that ATRA action for the cell growth inhibitory activity of EGCG is attributable to the enhancement of cell surface binding of EGCG via 67LR.

To determine the in vivo efficacy and safety of the combined treatment, mice were implanted with B16 cells and treated with EGCG and/or ATRA (Lee et al. 2010). Compared to treatment with a vehicle control, combined treatment significantly reduced the tumor volume over the duration of the study. The tumor volume and weight in mice treated with EGCG or ATRA alone did not differ from those in mice treated with the vehicle control. On the other hand, the mean tumor weight in the combination-treatment group was 40% less than that in the control group, indicating that ATRA intensifies the anti-tumor activity of EGCG. To examine whether 67LR are involved in the inhibition of tumor growth, we measured the expression of 67LR in the tumor cells. The 67LR levels in the tumor were increased upon oral administration ATRA, or combination of EGCG and ATRA.

RAR that binds to ligand ATRA form a heterodimer with RXRs and regulate the expression of specific genes (Martin et al. 2005). To investigate whether the ATRA-induced enhancement of 67LR expression is mediated through RARα, B16 cells were stably transfected with RARα shRNA expression vector that

Fig. 5. 67LR is a critical sensor molecule to respond to EGCG and mediates the beneficial activities of this phytochemical. Reprinted with permission from ILSI, 116, Tachibana, Green tea catechin sensing system, 6–13, Fig. 5, © 2014, ILSI Japan.
allows knockdown of RARα (Lee et al. 2010). Knockdown of RARα attenuated the ATRA-induced enhancement of 67LR expression. These results suggest that ATRA enhances 67LR expression through RARα.

To examine the participation of RAR in ATRA-mediated enhancement of EGCG-induced cell growth inhibition through 67LR, B16 cells were treated with the pan-RAR agonist TTNPB (Lee et al. 2010). TTNPB enhanced the protein levels and cell-surface levels of 67LR. Moreover, treatment with TTNPB enhanced EGCG-induced cell growth inhibition. The growth-suppressive effect by TTNPB treatment with EGCG and TTNPB was obviously reduced upon treatment with anti-67LR antibody. Collectively, these findings indicate that any compounds which activate RAR may be a candidate to enhance the antitumor activity of EGCG.

7-2. Low oxygen pressure

The microenvironment of malignant solid tumors is vastly different from normal tissues, and is characterized by extreme diversity in ionic strength, pH, the distribution of nutrients and O2 concentration. Increasing experimental evidence indicates that low O2 conditions have a profound impact on malignant progression and response to therapy. O2 conditions can be categorized as “low O2 (not hypoxia)”, “chronic hypoxia”, “acute hypoxia” and “cycling hypoxia”, according to causative factors and the duration of cancer cell exposure to hypoxic conditions. Activation of hypoxia-inducible factor 1 alpha (HIF-1α) in cancer cells induces the expression of various genes responsible for adaptation to hypoxic conditions and resistance to chemotherapy and radiation therapy. Pre-hypoxic regions (HIF-1-independent) are also known to exist within tumors. However, to date there are no studies regarding the effects of EGCG on cancer cells under hypoxic conditions. Therefore, we investigated the effect of O2 partial pressure on EGCG activity (Tsukamoto et al. 2012). We found that 67LR protein levels are reduced by exposure to low O2 levels (5%), without affecting the expression of HIF-1α. We also found that EGCG-induced anticancer activity is abrogated under low O2 levels (5%) in various cancer cells. Notably, treatment with the proteasome inhibitor, prevented down-regulation of 67LR and restored sensitivity to EGCG. In summary, 67LR expression is highly sensitive to O2 partial pressure, and the activity of EGCG can be regulated in cancer cells by O2 partial pressure.

8. Conclusions

Beneficial health effects by edible phytochemicals are now considered to be an inexpensive, readily applicable, and accessible approach to cancer control and management (Surh 2003), however, little is known about the mechanism of the chemopreventive action of most phytochemicals. The essence is the identification of the primary target and the demonstration of specific mechanisms of action in animal models and human tissues. Here we described that 67LR is a critical sensor molecule to respond to EGCG and mediates the beneficial activities of this phytochemical (Fig. 5). We also described that eEF1A, MYPT1, cGMP, and ASM are EGCG-sensing molecules for EGCG-induced cancer prevention in vivo, and these factors mediate unique signaling for cancer prevention triggered by physiological concentrations of EGCG. Our findings suggest that these are “master factors,” which determine the efficacy of cancer-preventive activity of EGCG and have important implications for development and use of EGCG as a cancer-chemopreventive agent. Probably, only a tumor with a high expression level of these “master factors” has sensitivity to physiological concentrations of EGCG, while lower expression of those molecules causes “EGCG-resistance”. Our results not only illustrate the mechanisms for the cancer-preventive activity of EGCG but should help in the design of new strategies to prevent cancer and underscore the importance of tailoring cancer therapy on the basis of tumor genotype.

More definitive information on the relationship between EGCG sensing-pathways and beneficial effects of EGCG ingestion will emerge from cohort studies and human intervention trials. We hope that this review will have wide-ranging implications, as many of the issues discussed here might also be applicable to studies of other dietary ingredients.

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