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Green tea polyphenol EGCG induces lipid-raft clustering and apoptotic cell death by activating protein kinase Cδ and acid sphingomyelinase through a 67 kDa laminin receptor in multiple myeloma cells

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EGCG [(−)-epigallocatechin-3-*O*-gallate], the major polyphenol of green tea, has cancer chemopreventive and chemotherapeutic activities. EGCG selectively inhibits cell growth and induces apoptosis in cancer cells without adversely affecting normal cells; however, the underlying molecular mechanism *in vivo* is unclear. In the present study, we show that EGCG-induced apoptotic activity is attributed to a lipid-raft clustering mediated through 67LR (67 kDa laminin receptor) that is significantly elevated in MM (multiple myeloma) cells relative to normal peripheral blood mononuclear cells, and that aSMase (acid sphingomyelinase) is critical for the lipid-raft clustering and the apoptotic cell death induced by EGCG. We also found that EGCG induces aSMase translocation to the plasma membrane and PKCδ (protein kinase Cδ) phosphorylation at Ser^{664}, which was necessary for aSMase/ceramide signalling via 67LR. Additionally, orally administered EGCG activated PKCδ and aSMase in a murine MM xenograft model. These results elucidate a novel cell-death pathway triggered by EGCG for the specific killing of MM cells.

Key words: acid sphingomyelinase (aSMase), apoptosis, (−)-epigallocatechin-3-*O*-gallate (EGCG), lipid-raft clustering, protein kinase Cδ (PKCδ).

INTRODUCTION

Tea (*Camellia sinensis* L.) is one of the most widely consumed beverages in the world. EGCG [(−)-epigallocatechin-3-*O*-gallate], the major green tea catechin present in the leaves, is primarily responsible for the health benefits attributed to green tea. EGCG possesses remarkable cancer chemopreventive and therapeutic potential against various cancer types. Moreover, EGCG is relatively safe [1], and green tea extract with 60 % EGCG has been approved by the US Food and Drug Administration as the first botanical drug [2].

EGCG has been studied extensively for its actions against cancer cells and related molecular mechanisms. The anti-cancer effect of EGCG is associated with apoptotic cell death and cell growth inhibition. Although many proteins bind EGCG with high affinity [3,4], the direct involvement of these proteins in the cancer-preventive activity of EGCG *in vivo* remains unclear.

EGCG induces apoptosis in several cancers, although often at higher concentrations (20–100 μM) than observed in plasma after drinking more than several cups of green tea (1 μM) [5]. EGCG-induced cell killing requires 67LR (67 kDa laminin receptor) expression in human AML (acute myeloid leukaemia) patient cells [6] and MM (multiple myeloma) patient cells [7]. In MM cells, silencing of 67LR with siRNA (small interfering RNA) blocked EGCG-induced apoptotic cell death, indicating that 67LR mediates the killing activity of EGCG, but the downstream mechanisms are still unclear.

67LR was originally identified as a non-integrin cell-surface receptor for the extracellular matrix molecule laminin [8–10]. Additionally, 67LR plays a major role as a cell-surface receptor in prion disorders and may be important in other neurodegenerative diseases, such as Alzheimer's disease [11]. A series of viruses (Sindbis virus, Dengue virus and adeno-associated virus) and the cytotoxic necrotizing factor 1 from *Escherichia coli* use 67LR for binding to mammalian cells [12,13]. The role of 67LR as a signalling mediator is still unclear, however.

Cytotoxins increase membrane fluidity [14,15] and induce apoptosis, but membrane-stabilizing agents block this increase, as well as cell-death induction in several cell models [16]. Previous studies have shown that anti-tumour ether lipid ET-18-OCH_3 induced apoptotic cell death in human leukaemic cells by inducing aggregation of Fas-containing lipid-raft membrane domains, indicating the role of Fas/lipid raft co-clustering for apoptosis [17,18]. Fas–FasL complexes enter initially into small membrane lipid rafts and induce a weak formation of the death-inducing signalling complex, generating ceramide by the aSMase (acid sphingomyelinase) to induce coalescence of elementary rafts (lipid-raft clustering) [19]. Thus sphingomyelinase and ceramide amplify death receptor signalling by inducing lipid-raft clustering.

aSMase-dependent ceramide as a lipid second messenger is a potent inducer of increasing membrane fluidity and apoptosis [15,20]. PKCδ (protein kinase Cδ) is required for aSMase activation in a breast cancer cells [21,22]. Additionally, PKCδ has an important role in apoptosis induced by oxidative stress, Fas ligand and paclitaxel [23–25].

In the present study we show that 67LR and EGCG form a novel cell death receptor/ligand system in MM cells. We show that: (i) specific killing of MM cells by EGCG results from inducing lipid-raft clustering via 67LR; (ii) EGCG-induced lipid-raft clustering requires aSMase activation and translocation to the

Abbreviations used: aSMase, acid sphingomyelinase; COX-2, cyclo-oxygenase 2; EC, epicatechin; EGCG, (−)-epigallocatechin-3-*O*-gallate; FBS, fetal bovine serum; FRET, fluorescence resonance energy transfer; HDAC7, histone deacetylase 7; 67LR, 67 kDa laminin receptor; MM, multiple myeloma; MYPT1, myosin phosphatase target subunit 1; PBMC, peripheral blood mononuclear cell; PKC, protein kinase C; shRNA, short hairpin RNA.

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cell membrane; and (iii) PKCδ mediates EGCG-induced aSMase activation following cell death. These results indicate that the 67LR/PKCδ/aSMase axis regulates EGCG-induced apoptotic cell death *in vivo*.

EXPERIMENTAL

Materials and antibodies

EGCG, desipramine, BODIPY®-C_{12}-sphingomyelin, catalase and PI (propidium iodide) were purchased from Sigma. Rottlerin was obtained from Calbiochem. Anti-aSMase antibody (H-181) was obtained from Santa Cruz Biotechnology, and anti-67LR (MLuC5) and phospho-PKCδ^{Ser664} antibodies were purchased from Abcam. Anti-67LR serum was obtained from a rabbit which was immunized with synthesized peptide corresponding to residues 161–170 of human 67LR. Fluorescein-tagged cholera toxin subunit B (CTx–Alexa Fluor® 488 and CTx–Alexa Fluor® 594) and annexin V–Alexa Fluor® 488 were obtained from Invitrogen. A PKC kinase activity assay kit was purchased from Assay Designs.

Patient samples and cell culture

Peripheral blood samples from patients with MM were from the VERITAS Corporation after informed consent was obtained. The purity of plasma cells (more than 80 %) was confirmed by monitoring cell-surface expression of CD38 and CD138. Mononuclear cells were also obtained with informed consent from peripheral blood donated by four healthy volunteers. Patient cells were cultured in RPMI 1640 medium supplemented with 10 % FBS (fetal bovine serum) and 2.5 ng/ml recombinant human IL (interleukin)-6 (R&D Systems). U266, ARH-77, RPMI8226 (human MM) and MPC-11 (mouse myeloma) cell lines were maintained in RPMI 1640 medium containing 10 % FBS. All cell lines were maintained in a state of exponential growth at 37 °C in humidified air with 5 % CO_2. To assess cell proliferation and viability, cells were plated in 24-well plates at 1×10^5 cells/ml, and they were treated with the indicated concentrations of EGCG for the indicated time periods in RPMI 1640 medium supplemented with 1 % FBS, 200 units/ml catalase and 5 units/ml SOD (superoxide dismutase) (Sigma).

Western blot analysis

Immunoblot analysis was performed as described previously [26].

Apoptosis assay

Apoptotic MM cells were detected using annexin V–Alexa Fluor® 488. Cells (1×10^6 cells/ml) were mixed with annexin V–Alexa Fluor® 488 and incubated in the dark for 15 min at 37 °C. Cells were then centrifuged (300 ***g*** for 3 min at 24 °C) and medium was replaced with binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). The cell suspension was placed on to a glass slide, covered with a coverslip, and viewed immediately using a fluorescence microscope equipped with a green filter.

Lipid-raft clustering assay

FRET (fluorescence resonance energy transfer) was used to evaluate lipid-raft clustering in the presence or absence of EGCG. U266 cells were stained with CTx–Alexa Fluor® 488 (as a donor) and CTx–Alexa Fluor® 594 (as an acceptor) for 1 h on ice. After treatment, cells were stimulated with EGCG at 37 °C for various times. The fluorescent images of lipid-raft staining were analysed using a fluorescence microscope (Keyence) and FRET signalling intensities were detected by flow cytometry analysis (FACSCalibur, Beckman Coulter; FlowJo software, Tomy Digital Biology).

TLC analysis

Cells were lysed in cell lysis buffer (pH 4.5) [26], incubated at 4 °C for 1 h, and then centrifuged at 15000 ***g*** for 15 min. The supernatant was incubated with substrate buffer containing 400 pmol of BODIPY®-C_{12}-sphingomyelin, 1 % Triton X-100 and 200 mM sodium acetate in distilled H_2O at 37 °C for 18 h. After incubation, the reaction was stopped by the addition of chloroform/methanol [2:1 (v/v)]. BODIPY®-sphingomyelin and BODIPY®-ceramide were separated from the remaining substrate by TLC using chloroform/methanol/water (65:25:4, by vol.) as a solvent. Ceramide levels were detected by UV irradiation and analysis was performed using KyPlot 4.0 software.

RNAi (RNA interference) by shRNA (short hairpin RNA)

Lentiviral vectors expressing non-targeting control shRNA and shRNAs targeting aSMase were purchased from Sigma–Aldrich. Lentivirus production, transduction and selection were performed according to the manufacturer's protocol.

Animals

SCID mice (CB-17) and BALB/c mice (Charles River Laboratories Japan) were kept in a 12-h light/12-h dark cycle (light on at 08:00 h) in an air-conditioned room (20 °C and 60 % humidity under specific pathogen-free conditions).

MM xenograft murine model

SCID mice were inoculated subcutaneously in the interscapular area with 5×10^6 ARH-77 cells in 100 μl of RPMI 1640 medium. BALB/c mice were inoculated subcutaneously in the interscapular area with 5×10^6 MPC-11 in 100 μl of RPMI 1640 medium. Following the appearance of palpable tumours, mice were administered orally or intraperitoneally with PBS alone or EGCG (20 mg/kg). After 4 h, tumours were excised and evaluated for the activity of cleaved caspase 3, PKCδ and aSMase. This experiment was performed in accordance with the law (#105) and notification (#6) of the Japanese government for the welfare of experimental animals. All of the procedures were approved by the Animal Care and Use Committee of Kyushu University and performed in strict accordance with institutional guidelines for handling laboratory animals.

Statistical analysis

The values of EGCG-induced pro-apoptotic activity in tumours were expressed as means ± S.E.M. compared with controls. The other values were expressed as means ± S.D. compared with controls. Statistical analysis was performed using Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

EGCG induces apoptotic cell death through a 67LR-dependent, but MYPT1 (myosin phosphatase target subunit 1)-independent, pathway in MM cells

Primary myeloma cells derived from an MM patient, MM U266 cells and normal PBMCs (peripheral blood mononuclear cells)

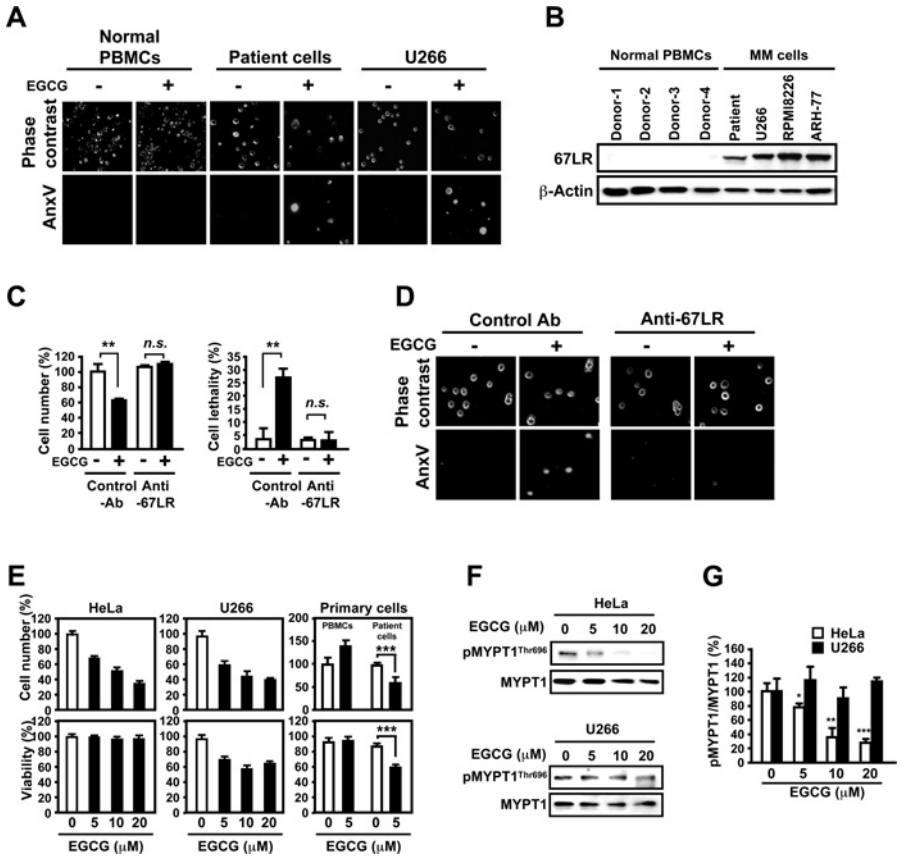

Figure 1 EGCG induces apoptotic cell death through 67LR, but via an MYPT1-independent pathway, in MM cells

(**A**) Normal PBMCs and primary MM cells derived from patient and myeloma U266 cells were treated with EGCG (5 μM) for 72 h, and apoptotic cells were viewed by fluorescence microscope using annexin V–Alexa Fluor® 488 and photographed by phase-contrast or by fluorescence emitted at 535 nm. (**B**) Western blot analysis of 67LR in each cell. (**C** and **D**) U266 cells were pretreated with either anti-67LR antibody or control antibody for 1 h before stimulation of EGCG (5 μM) for 72 h, and apoptotic cells were detected using the Trypan Blue exclusion method (**C**) or fluorescence microscope using annexin V–Alexa Fluor® 488 (**D**). (**E**) Cells were cultured in medium containing no EGCG or various concentrations of EGCG ranging from 5 to 20 μM for 72 h. The viability was evaluated using the Trypan Blue exclusion method. (**F** and **G**) Effect of EGCG on phosphorylation of MYPT1 at Thr^{696} in U266 cells and cervical carcinoma HeLa cells. Cells were treated with the indicated concentrations of EGCG for 24 h. MYPT1 phosphorylation levels were evaluated by Western blot analysis. Values are means $\pm$ S.D. for three samples. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t* test), compared with the control. Ab, antibody; AnxV, annexin V; n.s., not significant.

were sequentially treated with annexin V–Alexa Fluor® 488 after treatment with EGCG for 72 h, and apoptotic cells were evaluated (Figure 1A). EGCG treatment produced annexin V-positive cells in primary MM and U266 cells, but not in normal PBMCs. Next, we investigated whether EGCG-induced apoptotic cell death correlates with the expression of 67LR. All human myeloma cell lines (U266, RPMI8226 and ARH-77) and primary myeloma cells had higher levels of 67LR than normal PBMCs (Figure 1B). U266 cells were protected from EGCG-induced cell death when cells were treated with an anti-67LR antibody (Figures 1C and 1D), suggesting that 67LR plays a pivotal role in apoptotic death induced by EGCG.

EGCG inhibits cell proliferation of primary myeloma and U266 cells, as well as cervical carcinoma HeLa cells, but does not affect PBMCs (Figure 1E, top panels). EGCG also reduced the survival of U266 and primary MM cells, but did not affect the viability of HeLa cells (Figure 1E, bottom panels). We have reported that dephosphorylation of MYPT1 at Thr^{696} mediates the EGCG-induced growth inhibition of B16 melanoma cells and HeLa cells [26,27]. In immunoblotting, EGCG dose-dependently reduced phosphorylation of MYPT1 at Thr^{696} in HeLa cells, but did not affect phosphorylation in U266 cells (Figures 1F and 1G), indicating that this phosphorylation is not required for apoptosis induction in MM cells. Take together, these results suggest that EGCG induces apoptotic cell death through 67LR, but not via the MYPT1 pathway, in MM cells.

Apoptotic cell death induced by EGCG is dependent on lipid-raft clustering via 67LR

Increases in membrane fluidity and clustering of lipid rafts play crucial roles in apoptosis [16]. To examine the effect of EGCG on lipid-raft clustering, we evaluated FRET signalling after staining with CTx–Alexa Fluor® 488 and CTx–Alexa Fluor® 594 before stimulation with EGCG. Fluorescent images with lipid raft staining were analysed using a fluorescence microscope, and the FRET signalling intensities were detected by flow cytometry analysis. EGCG dose- and time-dependently increased lipid-raft clustering, but treatment with EC (epicatechin), which lacks biological activity, did not induce lipid-raft clustering in U266 cells (Figures 2A and 2B). 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 (Figure 2C). We next investigated the effect of cholesterol, a membrane-stabilizing agent, on EGCG-induced apoptosis.

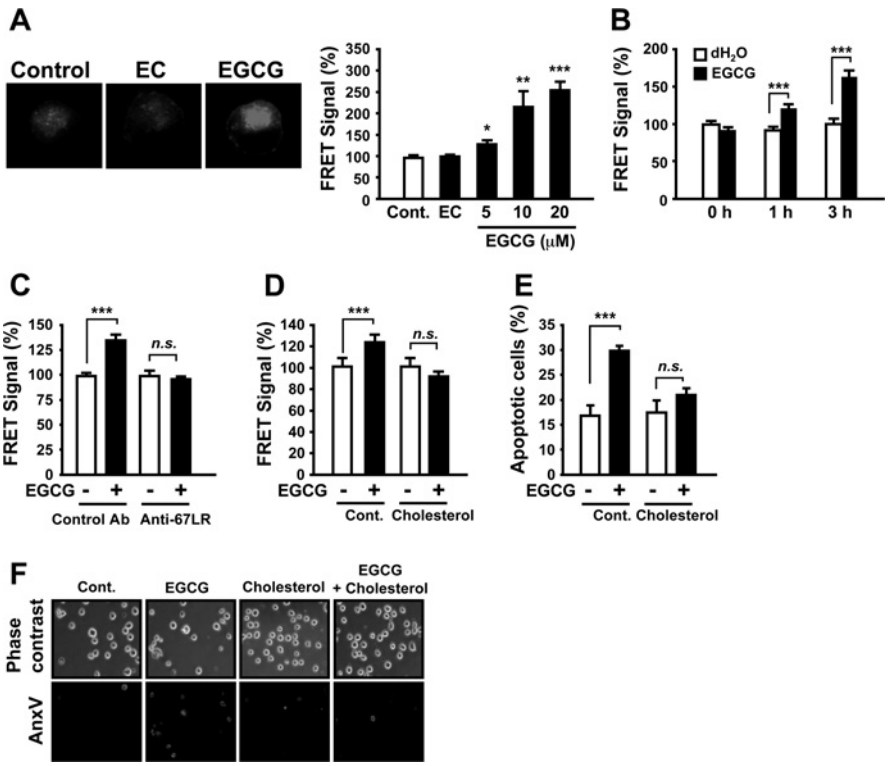

Figure 2 Apoptotic cell death induced by EGCG is dependent on lipid-raft clustering via 67LR

(**A** and **B**) U266 cells were stained with CTx–Alexa Fluor® 488 and CTx–Alexa Fluor® 594 for 1 h on ice. After treatment, cells were stimulated with EGCG (5–20 μM) or EC (10 μM) for 3 h (**A**) or treated with 10 μM EGCG for various times (**B**). The fluorescent images of lipid-raft staining were analysed using a fluorescence microscope after treatment with or without 10 μM EGCG and FRET signalling intensities were detected by flow cytometry in 500 cells. (**C**) After CTx–Alexa Fluor® 488 and CTx–Alexa Fluor® 594 staining, U266 cells were pre-incubated with 20 μg/ml anti-67LR antibody (MLuC5) or control antibody (mouse-IgM antibody) before treatment with 10 μM EGCG for 3 h. The lipid raft signalling intensities were detected by flow cytometry analysis in 500 cells. (**D**) After CTx–Alexa Fluor® 488 and CTx–Alexa Fluor® 594 staining, U266 cells were treated with 30 μg/ml cholesterol for 12 h before treatment with 10 μM EGCG for 3 h. The FRET signalling was detected in 500 cells. (**E** and **F**) U266 cells were treated with 10 μM EGCG for 72 h. After treatment, apoptotic cells were assessed by flow cytometry (**E**) and fluorescence microscopy (**F**) using annexin V–Alexa Fluor® 488. Values are means $\pm$ S.D. for three samples. $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t* test), compared with the control. Ab, antibody; AnxV, annexin V; Cont., control; dH2O, distilled water; n.s., not significant.

Exposure of myeloma cells to cholesterol inhibited lipid-raft clustering (Figure 2D) and apoptosis (Figures 2E and 2F), suggesting that the apoptotic activity of EGCG is caused by lipid-raft clustering.

EGCG activates the aSMase/ceramide pathway through 67LR

Lipid-raft clustering occurs after generation of ceramide by aSMase [14,15]. aSMase acts on membrane sphingomyelin to generate ceramide, which mediates cell death induced by diverse stimuli, such as ionizing radiation, chemotherapeutic agents and UV-A light. The expression of aSMase was abnormally elevated in all myeloma cell lines relative to normal PBMCs (Figure 3A). EGCG dose-dependently activated aSMase in U266 cells (Figure 3B), human MM cell lines and primary MM cells, but did not affect normal PBMCs from four different healthy donors (Figure 3C). Moreover, pretreatment with an anti-67LR antibody blocked EGCG-induced activation of aSMase, suggesting that 67LR mediates aSMase activation by EGCG (Figure 3D). ASMase 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 [15,28]. EGCG increased aSMase in the cell-membrane fraction (Figure 3E), and pretreatment with the anti-67LR antibody blocked this translocation (Figure 3F). Moreover, an aSMase-specific inhibitor, desipramine, blocked EGCG-induced ceramide production (Figure 3G). Taken together, these observations show that EGCG modulates the sphingolipid pathway through activating aSMase via 67LR.

aSMase is a critical mediator of EGCG-induced lipid-raft clustering and apoptotic cell death

Desipramine, an aSMase inhibitor, blocked EGCG-induced cell death in U266, MPC-11 and primary MM cells (Figure 4A), indicating that aSMase activity mediated this activity. Desipramine also blocked EGCG-induced apoptotic cell death and accumulation of sub-G_1 DNA (Figures 4B and 4C), as well as normalizing EGCG-induced lipid-raft clustering in U266 cells (Figure 4D). Transfection of U266 cells with an shRNA expression vector to reduce aSMase expression abolished EGCG-induced aSMase activation and apoptosis (Figure 4E). Transfection of RPMI8226 and ARH-77 cell lines with the same construct also prevented EGCG-induced cell death (Figure 4F). Collectively, these results suggest that aSMase is necessary for EGCG-induced lipid-raft clustering, leading to apoptotic cell death in MM cells.

EGCG-induced aSMase activation is dependent on PKCδ activation via 67LR

PKCδ is critical for the induction of apoptosis [23–25]. Therefore we tested whether PKCδ was involved in EGCG-induced activation of the aSMase/ceramide pathway. EGCG dose-dependently enhanced generic PKC activity (Figure 5A). EGCG

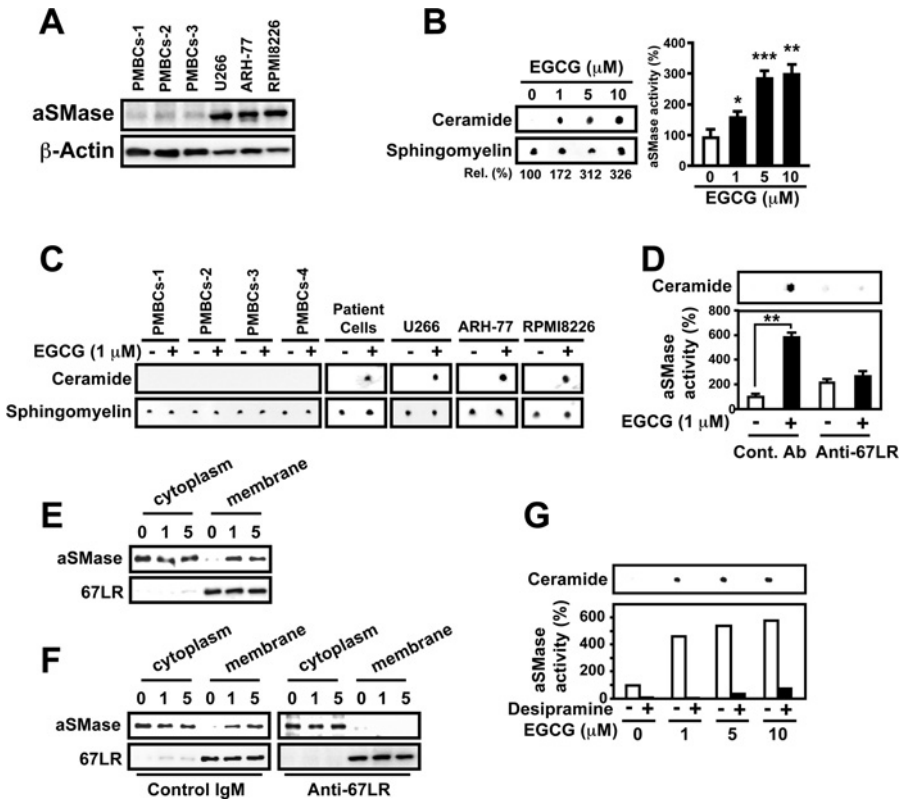

Figure 3 EGCG activates the aSMase/ceramide pathway through 67LR

(**A**) Western blot analysis of aSMase in each cell. (**B**) U266 cells were treated with the indicated concentrations of EGCG for 3 h, and cells were lysed with cell lysis buffer and incubated with BODIPY®-C_{12}-sphingomyelin. Ceramide levels were viewed by TLC analysis. (**C**) Normal PBMCs, three myeloma cell lines and myeloma patient samples were treated with EGCG for 3 h, and the aSMase activity was evaluated by the production of ceramide detected by TLC analysis. (**D**) U266 cells were treated with either anti-67LR antibody or control antibody for 1 h, and were treated with EGCG for 3 h. Activation of aSMase was assessed by ceramide production. (**E**) U266 cells were stimulated by EGCG for 3 h. Cytosolic and membrane fractions were prepared, and aSMase was detected by Western blot analysis. (**F**) U266 cells were pretreated with anti-67LR antibody or control antibody for 1 h before stimulation of EGCG for 3 h. After preparation of the membrane fraction, the protein expression levels of aSMase were detected by Western blot analysis. (**G**) U266 cells were pretreated with or without desipramine, a specific inhibitor of aSMase, for 3 h before EGCG treatment for 3 h, and aSMase activity was evaluated by ceramide production. Values are means ± S.D. for three samples. $^{**}P < 0.01$ (Student's *t* test), compared with the control. Ab, antibody; Cont., control; Rel., relative.

treatment of U266 cells for 5 min led to phosphorylation at Ser^{664} (Figure 5B) that was dose-dependent, but did not affect phosphorylation of Tyr^{155} and Thr^{507} (Figure 5C). Moreover, EGCG increased Ser^{664} phosphorylation in three MM cell lines and primary MM cells, but not in normal PBMCs derived from healthy donors (Figure 5D). 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 Ser^{664} was not observed in cells pretreated with the anti-67LR antibody (Figure 5E), suggesting that 67LR mediates EGCG-induced phosphorylation of PKCδ at Ser^{664}. Furthermore, treatment with the PKCδ-specific inhibitor rottlerin abolished the EGCG-induced activation of aSMase (Figure 5F). Silencing of aSMase in MM cells did not affect EGCG-induced PKCδ phosphorylation at Ser^{664} (Figure 5G). Overall, these results suggest that EGCG-induced aSMase activation is a secondary event that occurs after activation of PKCδ.

Orally or intraperitoneally administered EGCG activates caspase 3, PKCδ and aSMase in tumours

To evaluate the involvement of the PKCδ/aSMase 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 aSMase in tumour cells. ARH-77 cells were injected subcutaneously into SCID mice. Following tumour appearance (Figure 6A), the mice were treated with EGCG. Oral administration of EGCG promoted the cleavage of caspase 3, a key mediator of apoptosis, in tumour cells (Figure 6B). Moreover, immunofluorescence revealed that EGCG induced PKCδ phosphorylation at Ser^{664} in tumours (Figure 6C), 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 tumour cells (Figure 6D), as well as PKCδ phosphorylation at Ser^{664} and aSMase activation (Figures 6E and 6F). Orally administered EGCG produced similar effects on caspase 3, PKCδ and aSMase activation (Figures 6G, 6H and 6I). Consistent with the *in vitro* results, these results demonstrate that EGCG activates PKCδ and aSMase in MM cells *in vivo*, providing a molecular basis for EGCG-induced apoptosis in MM cells.

DISCUSSION

The therapeutic potential of EGCG calls for further study on the mechanism underlying its anti-cancer effects. EGCG has multiple potential mechanisms for inducing apoptosis, primarily based on

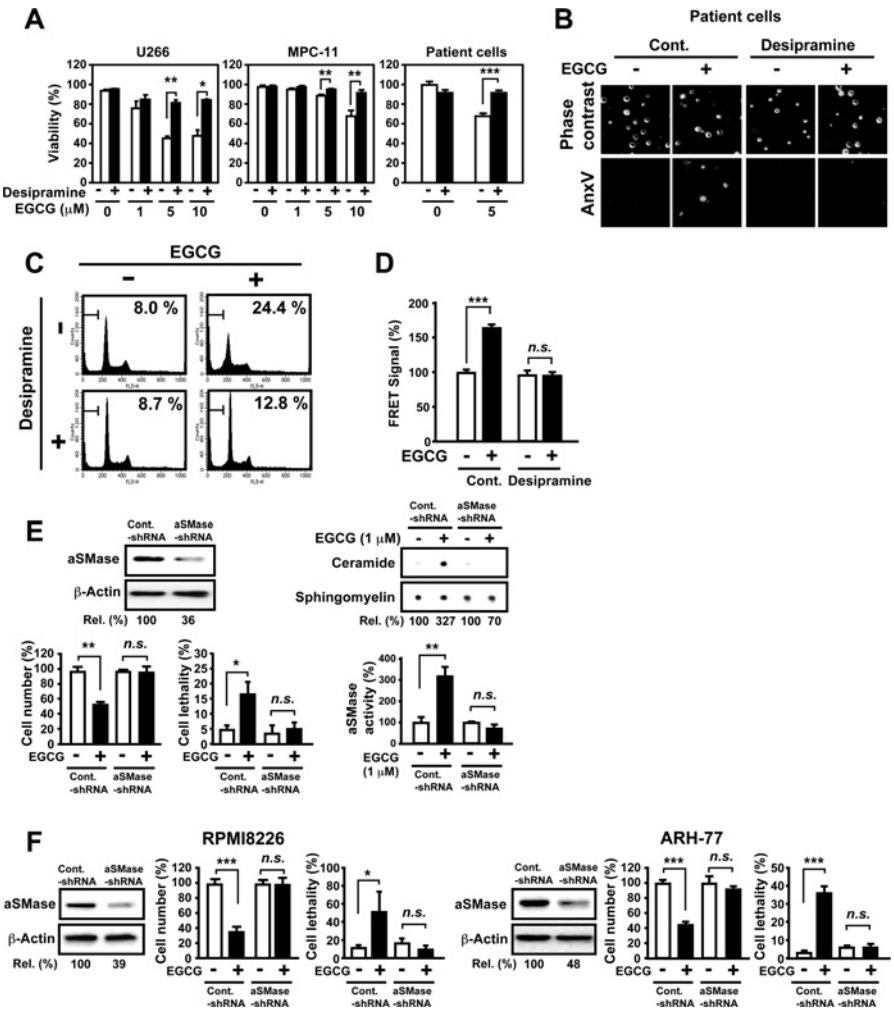

Figure 4 aSMase is a critical mediator for EGCG-induced lipid-raft clustering and apoptotic cell death

(**A**–**C**) Cells were treated with desipramine (5 μM) for 3 h and cultured in medium containing various concentrations of EGCG ranging from 1 to 10 μM for 72 h. (**A**) Desipramine protected from EGCG-induced cell death in U266 cells, MPC-11 and myeloma patient cells. The rate of cell lethality was assessed using the Trypan Blue exclusion method. (**B**) EGCG (5 μM)-induced apoptosis was evaluated by annexin V–Alexa Fluor® 488 in MM patient cells. (**C**) EGCG (5 μM)-induced accumulation of sub-G_1 DNA was assessed by flow cytometry in U266 cells. (**D**) After CTx–Alexa Fluor® 488 and –Alexa Fluor® 594 staining for 1 h, U266 cells were pretreated with desipramine for 3 h, and treated with or without EGCG for 3 h. The FRET signalling was detected in 500 cells. (**E**) Effect of silencing of aSMase on EGCG-induced aSMase activation and apoptotic cell death in U266 cells. Top left-hand panel: shRNA-mediated knockdown of aSMase. Top right-hand panel: the activity of ceramide production after treatment with EGCG for 3 h. Bottom panel: the rate of cell lethality assessed using the Trypan Blue exclusion method. (**F**) RPMI8226 and ARH-77 cells were transfected with the aSMase shRNA expression vector, and cultured in medium containing or not EGCG (5 μM) for 72 h. The rate of cell lethality was assessed using the Trypan Blue exclusion method. Values are means ± S.D. for three samples. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t* test), compared with the control. AnxV, annexin V; Cont., control; Rel., relative; n.s., not significant.

studies in cancer cell lines [29,30]. However, achievable levels *in vivo* are generally lower than those used in these *in vitro* studies, and *in vivo* mechanisms may therefore be different [30,31]. In the present study, we identified a novel cell-death pathway that mediates apoptosis induced by physiologically achievable concentrations of EGCG *in vivo*.

EGCG-induced cell death requires cell-surface 67LR in human acute myeloid leukaemia patient cells [6] and MM cells [7], whereas normal PBMCs do not express 67LR. However, the subsequent mechanisms involved after EGCG binds to 67LR are still unclear. We previously found that EGCG inhibited cell proliferation of HeLa cells through 67LR by reducing MYPT1 phosphorylation at Thr^{696}, leading to activation of myosin phosphatase and reduction of MRLC phosphorylation at Thr^{18}/Ser^{19} [27,32]. EGCG also inhibits cell proliferation via this mechanism in melanoma B16 tumours in mice [27]. However, in MM cells, EGCG did not reduce MYPT1 phosphorylation at Thr^{696}, suggesting that EGCG does not inhibit growth via the 67LR/MYPT1 pathway in MM cells. In contrast, EGCG did not induce apoptotic cell death in HeLa cells. MYPT1 promotes nuclear localization of HDAC7 (histone deacetylase 7), leading to the repression of the HDAC7 target *Nur77* and inhibiting apoptosis in thymocytes [33]. Activated MYPT1 might suppress the EGCG-induced apoptotic activity in HeLa cells. Additional studies will be required to determine why EGCG does not induce apoptosis in HeLa cells.

67LR signalling has been previously studied [34], and the receptor is involved in laminin-induced tumour cell attachment [35], migration [36] and shear stress-dependent endothelial nitric oxide synthase expression [37]. Although we do not know a direct role for 67LR in apoptotic cell death, it does function as a receptor for endogenous and exogenous molecules, including laminin, pathogenic prion protein, cytotoxic necrotizing factor 1 from *E. coli*, Dengue virus and Sindbis virus [10–13]. Interestingly, Sindbis virus infects mammalian cells via cell-surface 67LR and triggers apoptosis [12,38]. These results indicate a role for 67LR as a cell-death receptor.

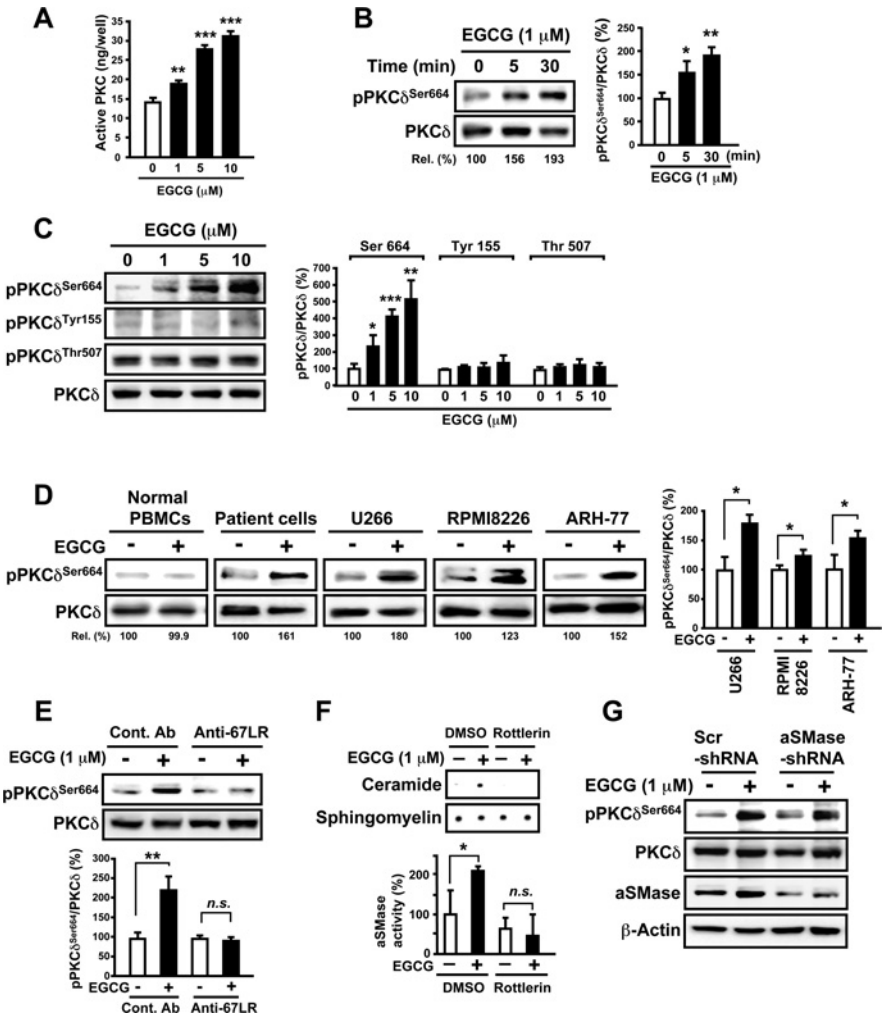

Figure 5 EGCG-induced aSMase activation is dependent on PKCδ activation via 67LR

(**A**) U266 cells were treated with the indicated concentrations of EGCG for 30 min, and PKC kinase activity was assessed using the PKC kinase activity assay kit. (**B**) After treatment with 1 μM EGCG for the indicated time, PKCδ phosphorylation at Ser^{664} levels were detected by Western blot analysis in U266 cells. (**C**) U266 cells were treated with the indicated concentrations of EGCG ranging from 1 to 10 μM for 30 min and the phosphorylation at different sites of PKCδ was assessed by Western blot analysis. (**D**) Normal PBMCs, three myeloma cell lines and myeloma patient samples were treated with or without 1 μM EGCG for 30 min and the phosphorylation levels of PKCδ were evaluated. (**E**) U266 cells were pretreated with either control antibody or anti-67LR antibody for 1 h before EGCG stimulation. After treatment with EGCG for 30 min, PKCδ phosphorylation levels were assessed by Western blot analysis. (**F**) U266 cells were pretreated with rottlerin for 3 h and cultured with or without 1 μM EGCG for 3 h. After lysis with cell lysis buffer and incubation with BODIPY®-C_{12}-sphingomyelin, ceramide production was evaluated by TLC analysis. (**G**) After treatment with 1 μM EGCG for 30 min in aSMase-knockdown U266 cells, the PKCδ phosphorylation levels were detected by Western blot analysis. Values are means $\pm$ S.D. for three samples. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t* test), compared with the control. Ab, antibody; Cont., control; Rel., relative; Scr, scrambled.

Cisplatin and other cytotoxins increase membrane fluidity [14,15] and induce apoptosis, but membrane-stabilizing agents block this increase as well as cell-death induction in several cell models. In MM cells, it has been demonstrated that the co-clustering of lipid raft and death receptor plays a critical role for apoptotic cell death [39,40]. We found that EGCG induced lipid-raft clustering and apoptotic cell death via 67LR, leading to death of cancer cells. Previous studies also showed that EGCG rapidly alters lipid organization in the plasma membrane [41–43].

Membrane fluidity is probably the most important physico-chemical property of cell membranes, including in response to cancer therapy [15,20]. Such alterations could involve membrane–drug interaction, modification of membrane lipid composition, membrane lipid peroxidation, cytoskeleton alteration or activation of sphingomyelinases, such as aSMase [19]. Several chemotherapeutic agents, such as doxorubicin and cisplatin, require aSMase to trigger apoptosis [14,44]. Furthermore, aSMase-dependent ceramide as a lipid second messenger potently increases membrane fluidity and apoptosis in colon cancer cells [15,18]. These findings suggest that the mechanisms by which EGCG induces apoptosis may be dependent on the aSMase/ceramide pathway elicited by chemotherapeutic agents, including cisplatin.

There are several reports highlighting the role of PKCδ both as a key target and effecter of aSMase activation. PKCδ regulates aSMase through phosphorylation at Ser^{508}. This phosphorylation appears to be essential for activation as well as translocation of aSMase to the plasma membrane [19,20]. Moreover, the *Prkcd*$^{-/-}$ (PKCδ) mouse shows a phenotype of suppressed apoptosis in response to UV-induced apoptosis through aSMase [45,46]. In this context, it is notable that EGCG activates PKCδ and induces translocation of the aSMase to the plasma membrane.

PKCδ can regulate apoptosis, either pro-apoptotic or anti-apoptotic, in different cell systems [47]. This dichotomous behavior might be, in part, controlled by phosphorylation at different

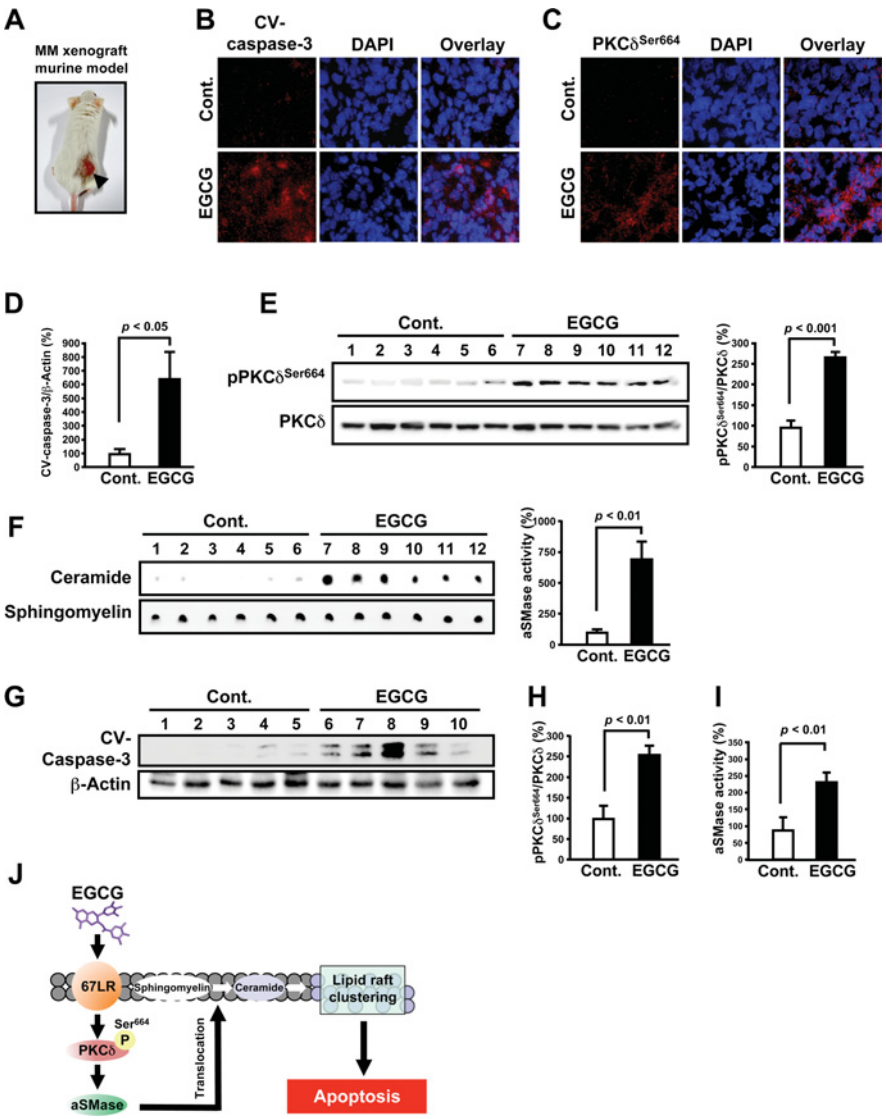

Figure 6 Orally or intraperitoneally administered EGCG activates caspase 3, PKCδ and aSMase in tumours

(**A**, **B** and **C**) SCID mice were inoculated subcutaneously in the interscapular area with ARH-77 cells. Representative appearance of tumours was photographed (**A**). The mice were administered orally with distilled H_2O or 20 mg/kg EGCG. After 4 h, tumours were excised and evaluated for cleaved caspase 3 (CV-caspase-3) expression (**B**) and PKCδ phosphorylation at Ser^{664} (**C**) by immunofluorescence analysis. (**D**–**I**) BALB/c mice were inoculated subcutaneously in the interscapular area with MPC-11 cells. Following appearance of tumours, the mice were treated orally (**D**, **E** and **F**) or intraperitoneally (**G**, **H** and **I**) with distilled H_2O alone (lanes 1–6 or 1–5) or 20 mg/kg EGCG (lanes 7–12 or 6–10). After 4 h, tumours were excised and evaluated for the activity of caspase 3 (**D** and **G**) and PKCδ (**E** and **H**) by Western blot analysis. The activation of aSMase was assessed as ceramide production activity by TLC analysis (**F** and **I**). Values are means $\pm$ S.E.M. for six (**D**, **E** and **F**) or five (**G**, **H** and **I**) mice. Significance was assessed using Student's *t* test. (**J**) Schematic representation of the EGCG-induced cell-death pathway through 67LR. Cont., control; DAPI, 4′,6-diamidino-2-phenylindole.

tyrosine residues of PKCδ by different kinases. For example, FTY720, a synthetic sphingosine immunosuppressant, promoted the phosphorylation of Tyr^{311}, leading to autophosphorylation at Thr^{507} and Ser^{664}, which presumably committed PKCδ to activating caspases through phosphorylation [48]. On the other hand, phosphorylation on Tyr^{155} promotes the anti-apoptotic effect of PKCδ in Sindbis virus-infected glioma cells [49]. Accordingly, the selective phosphorylation of PKCδ at Ser^{664}, but not Tyr^{155}, in EGCG-treated MM cells underscores the pro-apoptotic nature of this signalling pathway.

We found that EGCG activates the PKCδ/aSMase pathway through 67LR in MM cells, but not in normal PBMCs. This selective activity is attributed to 67LR expression, which is abnormally high in MM cells. The molecular mechanisms involved in EGCG-induced PKCδ activation via 67LR are not entirely clear. This issue is worthy of particular consideration to clarify the 67LR signalling elicited by EGCG.

MM cell lines (U266, RPMI8226 and ARH-77) examined in the present study showed different EGCG sensitivities. In these cells, the expression of the anti-apoptotic mediator COX-2 (cyclo-oxygenase 2) is markedly varied among the cell lines. ARH-77 cells constitutively express COX-2, whereas U266 and RPMI8226 are negative for it [50]. In addition, the expression levels of 67LR and aSMase in U266, RPMI8226 and ARH-77 cells are varied (Figures 1B and 3A). The distinct sensitivities to EGCG may be due to the difference of the expression levels of anti-apoptotic and pro-apoptotic proteins (e.g. COX-2, 67LR and aSMase) in the MM cell lines.

Collectively, the results of the present study indicate that 67LR, PKCδ, aSMase and lipid-raft clustering have major roles in

EGCG-induced apoptotic cell death. These results provide novel insights into the understanding of apoptotic cell-death signalling in MM cells. One of the practical implications of the present study is a new approach to develop cancer chemotherapy through cell-surface 67LR.

AUTHOR CONTRIBUTION

Shuntaro Tsukamoto, Keisuke Hirotsu, Motofumi Kumazoe, Yoko Goto and Hirofumi Tachibana designed the research. Shuntaro Tsukamoto, Keisuke Hirotsu, Motofumi Kumazoe, Yoko Goto, Kaori Sugihara, Takafumi Suda, Yukari Tsurudome, Takashi Suzuki and Hirofumi Tachibana performed the *in vitro* experiments with cell lines and the *in vivo* experiments. Shuntaro Tsukamoto, Keisuke Hirotsu, Motofumi Kumazoe, Yoko Goto and Hirofumi Tachibana contributed new reagents/analytical tools. Shuya Yamashita, Yoonhee Kim, Yuhui Huang, Koji Yamada and Hirofumi Tachibana advised on specific aspects of the experimental strategies. Shuntaro Tsukamoto and Hirofumi Tachibana wrote the paper.

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