ORIGINAL ARTICLE

Epigallocatechin-3-gallate potentiates curcumin's ability to suppress uterine leiomyosarcoma cell growth and induce apoptosis

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Abstract

Background Uterine leiomyosarcoma (LMS) has an unfavorable response to standard chemotherapeutic regimens. Two natural occurring compounds, curcumin and epigallocatechin gallate (EGCG), are reported to have anticancer activity. We previously reported that curcumin reduced uterine LMS cell proliferation by targeting the AKT–mTOR pathway. However, challenges remain in overcoming curcumin's low bioavailability.

Methods The human LMS cell line SKN was used. The effect of EGCG, curcumin or their combination on cell growth was detected by MTS assay. Their effect on AKT, mTOR, and S6 was detected by Western blotting. The induction of apoptosis was determined by Western blotting using cleaved-PARP specific antibody, caspase-3 activity and TUNEL assay. Intracellular curcumin level was determined by a spectrophotometric method. Antibody against EGCG cell surface receptor, 67-kDa laminin receptor (67LR), was used to investigate the role of the receptor in curcumin's increased potency by EGCG.

Results In this study, we showed that the combination of EGCG and curcumin significantly reduced SKN cell proliferation more than either drug alone. The combination inhibited AKT, mTOR, and S6 phosphorylation, and induced apoptosis at a much lower curcumin concentration than previously reported. EGCG enhanced the incorporation of curcumin. 67LR antibody partially rescued cell proliferation suppression by the combination treatment, but was not involved in the EGCG-enhanced intracellular incorporation of curcumin.

Conclusions EGCG significantly lowered the concentration of curcumin required to inhibit the AKT–mTOR pathway, reduce cell proliferation and induce apoptosis in uterine LMS cells by enhancing intracellular incorporation of curcumin, but the process was independent of 67LR.

Keywords Curcumin · EGCG · Uterine leiomyosarcoma · mTOR · AKT · 67-kDa laminin receptor

Introduction

Uterine leiomyosarcoma (LMS) is a relatively rare smooth-muscle tumor, and it accounts for approximately one-third of uterine sarcomas and 1.3% of all uterine malignancies [1]. The standard initial treatment is total abdominal hysterectomy with bilateral salpingo-oophorectomy, and debulking of the tumor if present outside the uterus [2]. Among the various chemotherapeutic regimens for advanced or recurrent uterine LMS, doxorubicin-based regimens show the largest response rates, ranging from 19 to 30% [3–5]. The combination of doxorubicin with other chemotherapeutic agents such as cyclophosphamide, vincristine and dacarbazine was able marginally to improve recurrence-free survival in case–control cohort studies, but caused more adverse events such as neutropenia and doxorubicin-related cardiomyopathies [6]. However, the improved survival for patients with LMS of the uterus observed in such studies was not observed in stringent randomized control trials [7]. Hence, the intractability of

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uterine LMS to conventional cytotoxic agents prompts us to develop new targeted therapies to replace or combine with the current regimens.

The PI3K (phosphotidyl-inositol-triphosphate)–AKT–(RAC-alpha serine-threonine-protein kinase)–mTOR (mammalian target of rapamycin) pathway is hyperactivated in LMS [8]. This growth-promoting pathway is a good approach for targeted therapies against LMS. Inhibitors targeting various components of this pathway are currently being tested at different stages of clinical trials, and have shown promising potential against uterine LMS [9]. In cells, two multiprotein complexes of mTOR have been identified: mTORC1 (mTOR complex 1) which consists of mTOR, raptor and mLST8, and is rapamycin-sensitive; and mTORC2 (mTOR complex 2) which is composed of mTOR, rictor, mLST8 and Sin1, and is rapamycin-insensitive [10]. As rapamycin is unable to inhibit rictor, mTOR inhibition by rapamycin via disruption of raptor enables a negative-feedback activation of AKT [11] by the rictor–mTOR complex [12].

In recent years, naturally occurring compounds have gained increasing attention in various clinical disciplines and disease. Green tea (Camellia sinensis) is a popular beverage worldwide. Among the constituents of green tea, commonly referred to as catechins, (−)-epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active component. Catechins including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and EGCG account for 30–40% of the dry weight of green tea [13, 14]. EGCG has various effects including anti-cancer [15, 16], anti-bacterial [17], anti-oxidative [18], anti-allergic [19], and anti-inflammatory effects [20, 21]. Another report showed that phosphorylated AKT (Ser473) levels in MDA-MB-231 human breast cancer cells and A549 lung cancer cells decreased noticeably under the treatment of 25 μM EGCG [22]. These data are consistent with the observation that EGCG has a direct inhibitory effect on AKT activity.

Curcumin is a phenolic compound present in the plant Curcuma longa, widely used in Asian cuisine. It has been reported that curcumin has cancer-preventive activity on several cancers, attributed to the modulation of numerous targets including transcription factors, receptors, kinases, cytokines, enzymes and growth factors [23]. Recently, we showed that curcumin reduced uterine LMS cell proliferation and induced apoptosis via repression of the AKT–mTOR pathway [24]. This study demonstrated that curcumin at a high concentration is able to inhibit both mTORC1 and mTORC2, contributing to curcumin's pro-apoptotic effect. However, curcumin's therapeutic potential is greatly limited by its low bioavailability.

To overcome the limitation in curcumin bioavailability, other studies have reported that combination treatment with EGCG and curcumin was synergistically cytotoxic and enhanced apoptosis in MDA-MB-231 human breast cancer cells [25] and chronic lymphocytic leukemia B cells [26]. Another constituent of green tea, EC, was also previously shown to enhance the uptake of curcumin into human lung cancer cell line PC-9 cells [27].

Taken as a whole, we hypothesized that EGCG and curcumin complement each other in reducing LMS cell proliferation and induce apoptosis by targeting the AKT–mTOR pathway. We also investigated the mechanism behind EGCG's ability to potentiate curcumin's anti-tumor property against uterine LMS cells by asking the question whether enhanced uptake of curcumin occurs in the presence of EGCG. Taking into consideration the recent discovery of 67-kDa laminin receptor (67LR) as a cell-surface EGCG receptor that is responsible for the anti-cancer activity of EGCG [28], we also performed in-vitro investigations to elucidate the role of this receptor in the enhanced effect as a result of combination treatment with EGCG and curcumin.

Materials and methods

Cell line and culture

The human uterine LMS cell line SKN [29] was purchased from Japan Health Sciences Foundation (Osaka, Japan). Cells were grown in complete HamF-12 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA) and maintained at 37°C in a humidified 5% CO_2 atmosphere.

Reagents

Curcumin was purchased from Wako Pure Chemical Industries, Ltd. and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at 1 M concentration as a stock solution that was stored at −20°C. (−)-Epigallocatechin-3-gallate (EGCG, 99% purity) was purchased from Sigma-Aldrich and dissolved in water at 20 mM concentration as a stock solution that was stored at 4°C and diluted in culture medium. Rapamycin was purchased from Cell Signaling Technology (Beverly, MA, USA) and dissolved in DMSO at 100 μM concentration as a stock solution that was stored at −20°C. Anti-67LR monoclonal antibody (MLuC5) was purchased from Abcam (Cambridge, UK), and isotype-matched anti-mouse IgM antibody (MOPC-104E) was purchased from Sigma-Aldrich and diluted in culture medium. Hank's buffered saline solution (HBSS) was purchased from Invitrogen (Carlsbad, CA, USA).

Anti-67LR antibody treatment

SKN cells were cultured in 100-mm dishes at 37°C in 5% CO_2 for 24 h before any treatment. To study the role of 67LR, cells were incubated with either anti-67LR monoclonal antibody (1 μg/ml), or control mouse IgM antibody (1 μg/ml) at 37°C in 5% CO_2 for 1 h before the addition of curcumin (10 μM) or the combination of curcumin (10 μM) with EGCG (150 μM) [30, 31].

Cell viability analysis

Analysis of cell viability was performed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay system (MTS assay) (Promega, Madison, WI, USA). SKN cells were seeded at 5,000 cells per well in a 96-well plate and incubated at 37°C in a humidified chamber with 5% CO_2 for 24 h before treatment with EGCG (100–200 μM), curcumin (5–10 μM), or their combination. All reagents were added and the MTS assay was performed according to the manufacturer's instructions. The results are based on at least three independent experiments. Results are expressed as the light absorbance at 490 nm.

Western blot analysis

Eighty to ninety percent confluent cells cultured for 24 h in 100-mm dishes containing 10 ml medium were exposed to different doses of reagents as indicated, before being harvested for Western blotting. Western blotting was performed as previously described [32]. Cell lysate proteins were fractionated by SDS-PAGE at 30 μg per well. For determination of AKT–mTOR pathway activity, the proteins were probed with AKT antibody, phospho-AKT (Thr308) antibody, mTOR antibody, phospho-mTOR (Ser2448) antibody, S6 ribosomal protein antibody and phospho-S6 ribosomal protein (Ser235/236) antibody at 4°C overnight. For detection of apoptosis, the blots were probed with human-specific cleaved PARP (Asp214) antibody. These antibodies were purchased from Cell Signaling Technology. The loading control, β-actin antibody, was purchased from Sigma-Aldrich.

Determination of apoptosis

To test whether curcumin, EGCG, or the combination induce apoptosis, we used the CaspACE™ Assay System (Promega). Vehicle control (0.01% DMSO), curcumin (10 μM), EGCG (100–200 μM) or their combination were added to the cells in a 100-mm dish and incubated for 6 h. Cells were collected and stored at −80°C. Whole-cell lysates were prepared in a buffer containing 20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA,

1 mM DTT, 0.1% protease inhibitor cocktail (Sigma-Aldrich) and 0.5% Nonidet P-40 (Sigma-Aldrich). 40 μg protein from each sample was used to measure caspase-3 activity and other reagents were added following the manufacturer's instructions.

TUNEL staining using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) was performed on adherent SKN cells cultured in an 8-well Lab-Tek™ chamber slide (Thermo Scientific, Rochester, NY, USA). SKN cells were seeded at 5,000 cells per well and incubated at 37°C in a humidified chamber with 5% CO_2 for 48 h. The cells were then treated with vehicle control (0.01% DMSO), curcumin (10 μM), EGCG (100–200 μM) or their combination for 48 h. TUNEL staining was performed according to the manufacturer's protocol. At least three independent microscopic fields were observed for each sample and stained nuclei were counted as positive.

Determination of intracellular curcumin

The intracellular curcumin level was determined by a spectrophotometric method. Cells were treated with EGCG (50–200 μM), curcumin (10 μM), or their combination. Cells were collected after 1 h, and then washed thoroughly with cold HBSS. The cells were resuspended in cold lysis buffer 0.1% Triton X-100 (Sigma-Aldrich) and 0.1% Nonidet P40 in HBSS, and disrupted by sonication and centrifuged for 10 min at 14,000g. The resulting supernatant fractions (50 μg protein) were diluted with HBSS to a final volume of 100 μl. The curcumin level in the fractions was determined by measuring the absorbance at 427 nm [27, 33].

Results

Combination treatment with EGCG and curcumin reduces uterine LMS cells viability more than treatment with either drug alone

We previously showed that curcumin inhibited uterine LMS cell proliferation in a dose-dependent manner [24]. To evaluate the enhanced effect of EGCG and curcumin combination, we performed MTS assay on SKN cells treated with vehicle control (0.01% DMSO), EGCG, curcumin, or the combination of EGCG and curcumin for 72 h (as illustrated in Fig. 1). Curcumin alone (5–10 μM) or EGCG alone (50–150 μM) did not significantly reduce the cell proliferation compared with vehicle control. Combination treatment of SKN cells with EGCG and curcumin produced a greater degree of cytotoxicity than treatment with either compound alone. Specifically, combination treatment with EGCG (200 μM) and curcumin (10 μM)
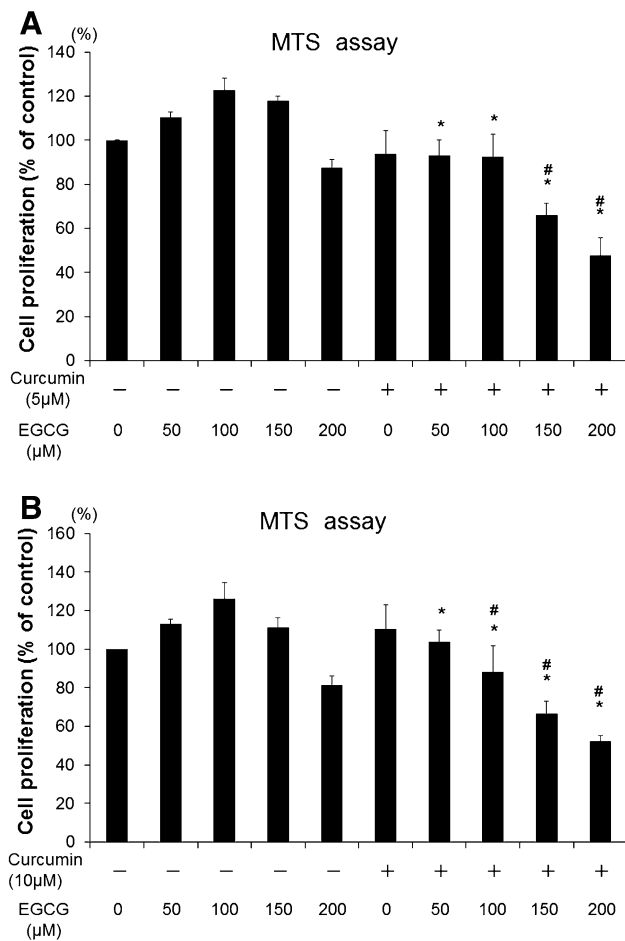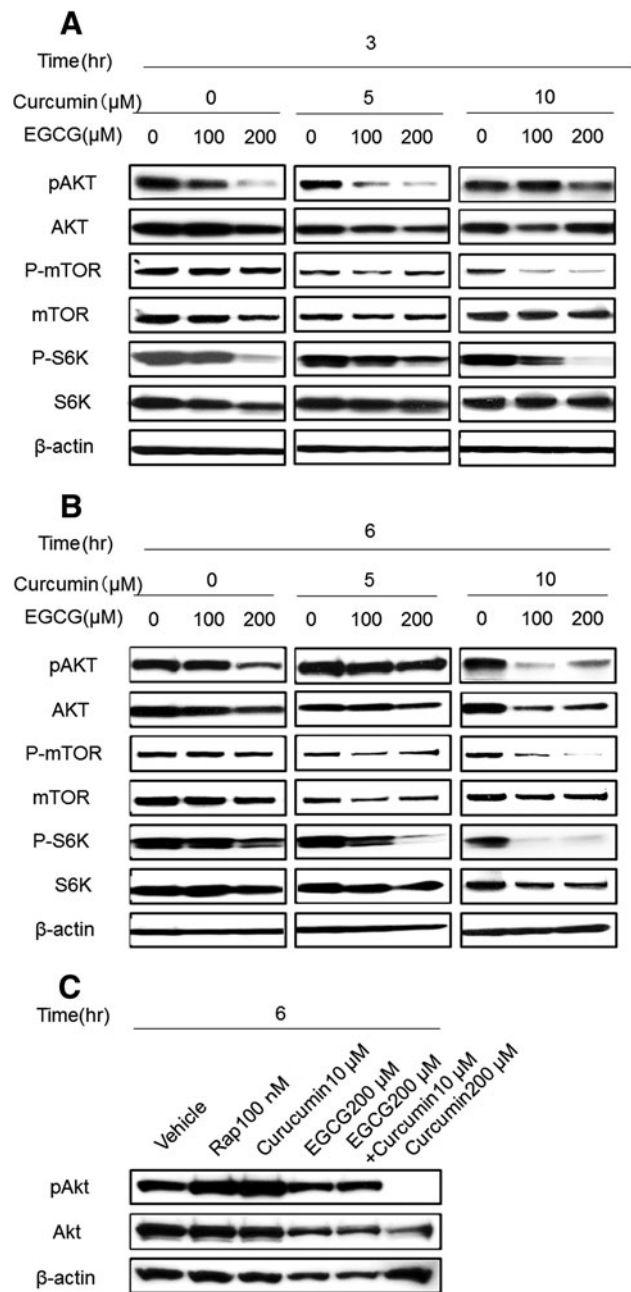
Fig. 1 Cell viability following treatment with EGCG and curcumin. **a** SKN cells were treated with either EGCG (50–200 μM), curcumin (5 μM), or a combination of the two for 72 h. **b** SKN cells were treated with either EGCG (50–200 μM), curcumin (10 μM), or a combination of the two for 72 h. Vehicle control cells were treated with DMSO (0.01% v/v). Cell viability was assessed by MTS assay. Independent *t* tests were used for all statistical comparisons. *$p < 0.05$: the effects of the combination are significantly different from those of EGCG alone. #$p < 0.05$: the effects of the combination are significantly different from those of curcumin alone


Fig. 2 Combination treatment with EGCG and curcumin reduce SKN cell viability via AKT–mTOR pathway inhibition. **a** SKN cells were treated with either EGCG (100–200 μM), curcumin (5 or 10 μM), or a combination of EGCG and curcumin for 3 h. **b** SKN cells were treated with either EGCG (100–200 μM), curcumin (5 or 10 μM), or a combination of EGCG and curcumin for 6 h. Vehicle control cells were treated with DMSO (0.01% v/v). **c** SKN cells were treated with either rapamycin (100 nM), EGCG (200 μM), curcumin (10 or 200 μM), or a combination of EGCG (200 μM) and curcumin (10 μM) for 6 h. Vehicle control cells were treated with DMSO (0.1% v/v). Phospho-AKT, AKT, phospho-mTOR, mTOR, phospho-S6 and S6 antibodies were used to detect the effect of treatment with a combination of EGCG and curcumin on the AKT–mTOR pathway

significantly decreased cell numbers by 47.5 ± 2.7% of control, while EGCG (200 μM) alone decreased cell numbers by 18.5 ± 4.8% of control (as shown in Fig. 1b). These results showed that combination treatment with EGCG and curcumin reduced uterine LMS cells viability more than treatment with either drug alone.

Combination treatment of EGCG and curcumin inhibits AKT and mTOR in a complementary fashion

To elucidate the potential mechanism behind the efficacy of the combination treatment, cell extracts were analyzed for changes in the protein expression and phosphorylation levels in the AKT–mTOR pathway. As shown in Fig. 2a, b,

EGCG alone reduced AKT phosphorylation after 3 and 6 h of treatment in SKN cells. 200 μM EGCG was also more effective than 100 μM EGCG. However, the phosphorylation of mTOR did not seem to be affected. Curcumin alone, at low concentrations, i.e. 5 and 10 μM, was not able to suppress the activity of either AKT or mTOR, and S6 remained highly phosphorylated. In contrast with individual treatment by EGCG, mTOR phosphorylation was reduced in combination with curcumin at 10 μM starting from 3 h of treatment (Fig. 2a); and even at as low as 5 μM curcumin, mTOR phosphorylation was reduced at 6 h of treatment (Fig. 2b). After 6 h exposure, EGCG combined with 10 μM curcumin not only reduced mTOR phosphorylation, but the phosphorylation of both AKT and S6 was also drastically reduced. At 3 h of 10 μM curcumin combined with EGCG, and 6 h of 5 μM curcumin combined with EGCG, AKT phophorylation seemed to be increased. However, at 6 h of 10 μM curcumin combined with EGCG, AKT phosphorylation decreased again. To investigate the effect of various treatments on the AKT–mTOR pathway, we performed an experiment as in Fig. 2c. Inhibition of mTOR alone as demonstrated by using 100 nM rapamycin for 6 h resulted in an increase in AKT phosphorylation, which was similar to that with curcumin 10 μM alone. EGCG 200 μM alone, as shown previously in Fig. 2a, b, could reduce AKT phosphorylation. At extreme concentrations such as curcumin 200 μM, AKT phosphorylation was entirely abolished at 6 h. The combination treatment with EGCG 200 μM and curcumin 10 μM was able to inhibit AKT reactivation compared with treatment using curcumin 10 μM alone.

Combination treatment induces apoptosis in uterine LMS cells more than treatment with either drug alone

We previously reported that curcumin induces apoptosis in uterine LMS cells; we then examined whether the combination treatment induces apoptosis. Early apoptotic events, as shown by an increase in PARP cleavage and caspase-3 activity, are increased even with EGCG 200 μM alone, but not with a lower EGCG concentration of 100 μM (as shown in Fig. 3). Curcumin at low concentrations, 5 and 10 μM, was not able to induce PARP cleavage (Fig. 3a, b) and caspase-3 activity (Fig. 3c). However, PARP cleavage and an increase in caspase-3 activity were observed from 100 μM EGCG combined with curcumin. As shown in Fig. 4, although EGCG alone at 200 μM was sufficient to induce early apoptotic events, late apoptosis as defined by DNA fragmentation (TUNEL assay) did not occur as efficiently as with combination treatment with EGCG and curcumin. Curcumin at 10 μM alone also did not induce DNA fragmentation effectively. In contrast, when curcumin 10 μM was used in combination with 200 μM EGCG,
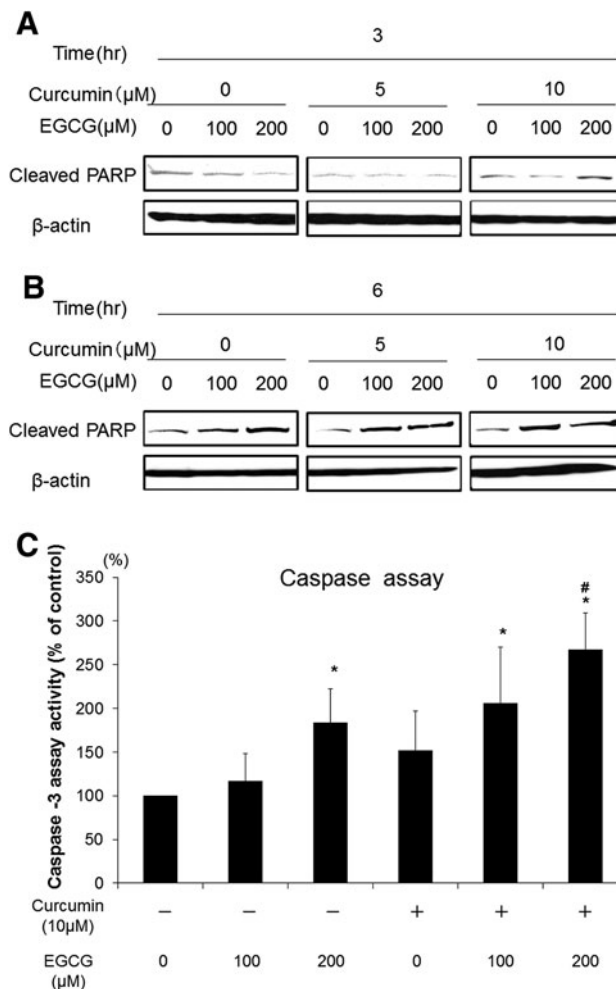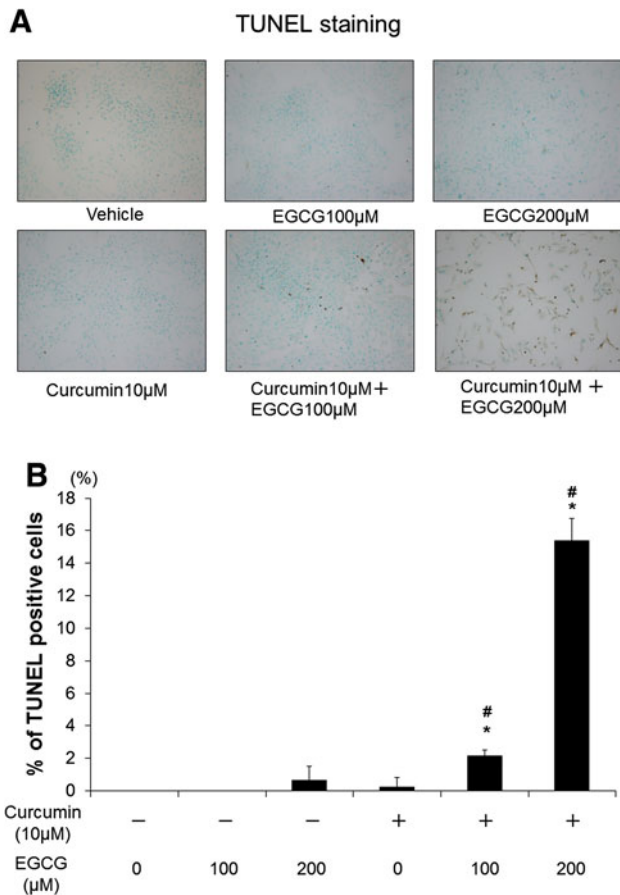

Fig. 3 Determination of apoptosis induction by EGCG and curcumin in SKN cells. a SKN cells were treated with either EGCG (100–200 μM), curcumin (5 or 10 μM), or a combination of EGCG and curcumin for 3 h. b SKN cells were treated with either EGCG (100–200 μM), curcumin (5 or 10 μM), or a combination of EGCG and curcumin for 6 h. Induction of apoptosis was determined using human-specific cleaved PARP antibody. Vehicle control cells were treated with DMSO (0.01% v/v). c Caspase-3 activity was measured in SKN cells treated with either EGCG (100–200 μM), curcumin (10 μM), or a combination of the two for 6 h using the luminescence-based caspase-3 kit. Vehicle control cells were treated with DMSO (0.01% v/v). Independent t tests were used for all statistical comparisons. *$p < 0.05$: the effects of the combination are significantly different from those of vehicle control. #$p < 0.05$: the effects of the combination are significantly different from those of curcumin alone

ten times more DNA fragmentation occurred, as shown in the TUNEL assay, compared with individual treatment using either 200 μM EGCG or 10 μM curcumin alone.

EGCG enhanced the incorporation of curcumin in uterine LMS cells

Because combination treatment with EGCG and curcumin reduces uterine LMS cell viability and induces apoptosis

A TUNEL staining

Vehicle — EGCG100μM — EGCG200μM

Curcumin10μM — Curcumin10μM+EGCG100μM — Curcumin10μM + EGCG200μM

B

% of TUNEL positive cells

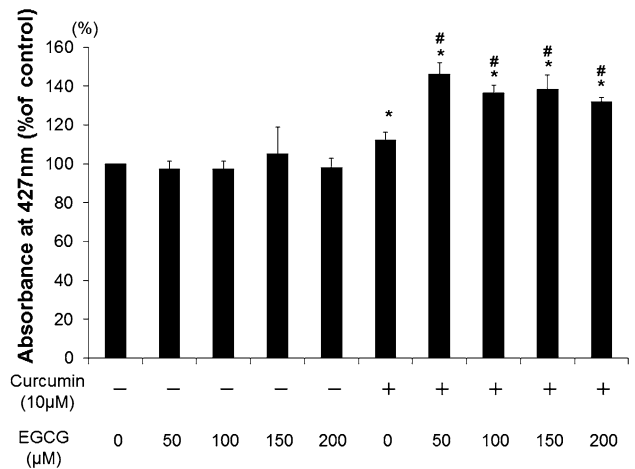| Curcumin (10μM) | − | − | − | + | + | + |
| EGCG (μM) | 0 | 100 | 200 | 0 | 100 | 200 |

Fig. 4 Combination treatment with EGCG and curcumin induces apoptosis in SKN cells. **a** TUNEL staining to detect DNA fragmentation in SKN cells. SKN cells were treated with either EGCG (100–200 μM), curcumin (10 μM), or a combination of the two for 48 h. DMSO (0.01% v/v) was used as vehicle control. *Dark nuclei* indicate positive staining. **b** Quantification of TUNEL-positive SKN cells. Independent t tests were used for all statistical comparisons. *$p < 0.01$: the effects of the combination are significantly different from those of EGCG alone. #$p < 0.001$: the effects of the combination are significantly different from those of curcumin alone

more than treatment with the individual drug alone, we examined whether EGCG enhances the incorporation of curcumin in SKN cells. As shown in Fig. 5, the combination of EGCG (50–200 μM) with curcumin (10 μM) significantly increased the intracellular curcumin ($p < 0.01$) compared with curcumin alone. These results indicate that EGCG enhances the uptake of curcumin in uterine LMS cells.

67LR antibody partially rescued the reduction of cell viability due to the combination treatment in uterine LMS cells

Recently, 67LR has been identified as a cell-surface receptor for EGCG. It was previously reported that anti-67LR blocking antibody can block the binding of EGCG to

Fig. 5 The intracellular curcumin level was measured in SKN cells treated with either EGCG (50–200 μM), curcumin (10 μM), or a combination of the two for 1 h by a spectrophotometric method. DMSO (0.01% v/v) was used as vehicle control. Independent t tests were used for all statistical comparisons. *$p < 0.01$: the effects of the combination are significantly different from those of vehicle control. #$p < 0.01$: the effects of the combination are significantly different from those of curcumin alone
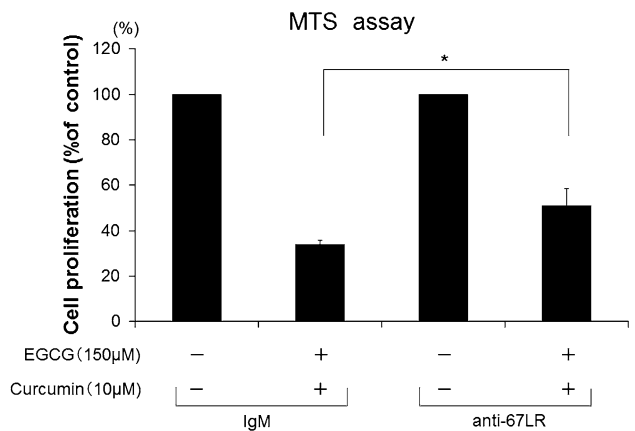
Fig. 6 67LR antibody partially rescued the reduction of cell viability due to combination treatment of SKN cells with EGCG and curcumin. SKN cells were treated with either vehicle control (0.01% DMSO) or EGCG (150 μM) and curcumin (10 μM) with 67LR antibody (1 μg/ml) or anti-mouse IgM (1 μg/ml) for 72 h. Cell viability was assessed by MTS assay. Independent t tests were used for all statistical comparisons. *$p < 0.05$: the effects of the combination treatment with 67LR antibody are significantly different from the combination treatment with anti-mouse IgM antibody

67LR, thereby inhibiting the action of EGCG [28, 30]. To study the effect of 67LR in uterine LMS, we performed MTS assay on SKN cells treated with vehicle control (0.01% DMSO) or EGCG (150 μM) and curcumin (10 μM) with monoclonal 67LR antibody or anti-mouse IgM antibody. As shown in Fig. 6, treatment with anti-67LR antibody reduced cell viability by 48.9 ± 7.4% of control, while treatment with anti-mouse IgM antibody

reduced cell viability by $65.8 \pm 1.5\%$ of control ($p < 0.05$). These results showed that anti-67LR antibody partially rescued the repressive effect of the combination treatment on SKN cell viability.

We further examined whether 67LR was involved in the enhanced incorporation of curcumin with EGCG in SKN cells. EGCG increased the incorporation of curcumin in SKN cells significantly, even in the presence of anti-67LR antibody (data not shown). These results indicated that EGCG-enhanced incorporation of curcumin was independent of 67LR.

Discussion

In this study, we showed that the combination treatment of EGCG and curcumin is synergistically cytotoxic toward uterine LMS cells in vitro. Combination treatment with EGCG and curcumin reduced uterine LMS cells viability more than treatment with either drug alone.

In our previous study, we found that curcumin reduces uterine LMS cell viability in a dose-dependent manner. In particular, curcumin achieved almost 80% inhibition of cell growth at 200 μM [24]. However, in clinical settings, it is very hard to achieve such a high serum level of curcumin because of its low efficiency of absorption. Therefore, many efforts have been made to increase the bioavailability of curcumin. For example, combination with piperine has been used to decrease the glucuronidation of curcumin and excretion; however, this combination was ineffective for the repression of tumor growth [34]. Other reports have shown that the combination of EGCG and curcumin is synergistically cytotoxic toward human breast cancer cells [25], chronic lymphocytic leukemia B cells [26] and familial colon cancer in vivo model [35]. These reports and our results suggest that in combination with EGCG, lower doses of curcumin can be developed as a treatment model to inhibit uterine LMS cells proliferation in vivo.

We also showed that combination treatment with EGCG and curcumin reduce uterine LMS cell viability via AKT–mTOR pathway inhibition more than treatment with either drug alone. In our previous study [24], curcumin 100 μM did not reduce the phosphorylation of mTOR; however, our present result showed that curcumin, at concentrations as low as 10 or 5 μM, with EGCG was able to reduce the phosphorylation of mTOR. Therefore, our studies demonstrated that the effective dose of curcumin required to induce significant tumor suppression could be substantially reduced when combined with EGCG.

EGCG alone is able to reduce AKT phosphorylation [22]. Our results were consistent with these observations, and 200 μM EGCG indeed seemed to directly inhibit AKT phosphorylation (Fig. 2). However, treatment with EGCG alone was limited by its inability to reduce the phosphorylation of mTOR. Curcumin at low concentrations, i.e. 5 and 10 μM, was not able to inhibit the AKT–mTOR pathway. On the other hand, when combined with EGCG, intracellular curcumin increased so much that its effect on mTOR became apparent.

Previously, we showed that at a lower concentration, curcumin was more effective at inhibiting mTORC1, whereas at 200 μM curcumin, not only mTORC1, but even mTORC2 was inhibited, as demonstrated by the total abolition of AKT phosphorylation [24]. The effect of the combination treatment (10 μM curcumin and 200 μM EGCG) on AKT, mTOR and its downstream target S6 cannot be explained by EGCG's direct inhibitory effect on AKT alone because of its limited effect on mTOR phosphorylation (Fig. 2). Instead we reasoned that EGCG increased curcumin's intracellular concentration (Fig. 5), bringing about the complementary effect from a high intracellular curcumin concentration that could eventually inhibit mTORC2 in addition to mTORC1 inhibition, as in the case of 200 μM curcumin (as shown in Fig. 2c). Unlike mTORC1-specific inhibition, as exemplified by rapamycin and perhaps 10 μM curcumin, which may result in AKT negative-feedback reactivation (Fig. 2c) [36], a higher intracellular concentration of curcumin coupled with EGCG's inherent anti-tumor potential resulted in a double effect that inhibited the AKT–mTOR pathway.

We demonstrated that combination treatment was able to induce apoptosis in uterine LMS cells (Figs. 3, 4). Apoptosis is a complex process that involves activation of the caspase enzymes and poly-ADP ribose polymerase (PARP) cleavage in the early stage [37], and culminates in DNA fragmentation or laddering in the final stage [38]. In our previous study [24], curcumin up to 100 μM in concentration was not able to induce PARP cleavage effectively at 3 or 6 h; however, our present result showed that curcumin, at concentrations as low as 5 or 10 μM with EGCG 200 μM at 6 h, was able to induce PARP cleavage effectively. In TUNEL staining, curcumin 10 μM with EGCG 200 μM induced DNA fragmentation significantly ($p < 0.001$, $15.4 \pm 1.4\%$), which was higher than the TUNEL staining at 100 μM curcumin ($10.5 \pm 3.2\%$) [24]. Although 200 μM EGCG alone could induce early apoptosis, combination treatment with curcumin was necessary for the induction of late apoptosis (DNA fragmentation), further supporting the case for combination treatment using EGCG and curcumin.

Our results showed that the presence of EGCG significantly increased the incorporation of curcumin into the cells (Fig. 5). It has also been reported that the combination of EC and curcumin significantly increased the intracellular curcumin [27], but the precise mechanisms of this enhancement remain unknown. As 67LR has recently been

identified as a cell-surface receptor for EGCG that mediates the anti-cancer action of EGCG, we decided to block the receptor using 67LR antibody. Our results demonstrated that 67LR antibody partially rescues the reduction of cell viability due to this combination treatment (Fig. 6), corroborating the results of previous reports on 67LR's role in EGCG-mediated anti-tumor activity. We went one step further by investigating 67LR's involvement in curcumin's enhanced incorporation by EGCG. But this pathway seemed not to be involved in the EGCG-induced enhancement of curcumin incorporation (data not shown).

The limitation of this study is that these findings were based on in-vitro experiments. Further studies are needed to show the direct anti-proliferative effect of this combination treatment on uterine LMS growth in an in-vivo model. We are now trying to study the effect of the treatment using an in-vivo model. This may aid in the development of novel therapeutic strategies for uterine LMS.

Conflict of interest The authors have no conflict of interest.

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