Celastrol enhances TRAIL-R2-mediated apoptosis and cytotoxicity in human renal cell carcinoma cells in caspase-dependent manner

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Abstract. Celastrol is a triterpene phytochemical known to possess anti-inflammatory, antioxidant and anticancer properties. The present study investigated the effects of celastrol on tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2)-mediated apoptosis and cytotoxicity in human renal cell carcinoma (RCC) cells when administered in combination with lexatumumab, a human monoclonal agonistic antibody that specifically recognizes TRAIL-R2. Cytotoxicity was determined by colorimetric assays of cell viability using cell counting kit-8. The activation of caspases was assessed by quantitative colorimetric assays using caspase-specific kits. Celastrol significantly enhanced lexatumumab-induced apoptosis and cytotoxicity in RCC cells. An enhanced effect was also achieved when the duration of treatment with lexatumumab and celastrol was reduced from 24 to 6 h. The expression of TRAIL-R2 was also remarkably increased by celastrol. Combined treatment with lexatumumab and celastrol significantly triggered activation of the caspase cascade, including caspase-8, -9, -6, and -3, downstream molecules of death receptors. Furthermore, the cytotoxicity induced by that combination was significantly suppressed by the DR5:Fc chimeric protein, as well as specific inhibitors of caspase-8, -9, -6 and -3. Taken together, these results indicated that celastrol enhances both TRAIL-R2-mediated apoptosis and cytotoxicity by upregulating TRAIL-R2 and activating the caspase cascade, indicating the possibility of using it in combination with lexatumumab as an innovative therapeutic strategy for treating RCC.

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Introduction

Renal cell carcinoma (RCC), the prevailing kidney malignancy in adults, has been shown to be resistant to chemotherapy and radiation therapy (1). Though the use of targeted agents, such as sunitinib, axitinib, temsirolimus, pazopanib and cabozatinib has been reported to notably prolong survival in patients with advanced RCC, the responses induced by these drugs are only transient (2,3). The recent introduction of immunotherapy utilizing immune checkpoint inhibitors, including nivolumab, pembrolizumab, ipilimumab and avelumab, holds promise for providing significant antitumor activity, as well as enduring responses, in patients with advanced RCC. However, the complete response rates induced by these drugs are limited, ranging from 4-10% (4,5). Another related concern is the occurrence of immune-related adverse events, as these can have effects on nearly all organs with varying frequency and severity, including hypophysitis, thyroiditis, hepatitis, interstitial pneumonitis, colitis and interstitial nephritis (6). Consequently, a pressing need for the development of innovative and effective therapeutic approaches for metastatic RCC has become evident.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a TNF superfamily member, is promising as an effective anticancer agent due to its capacity to selectively induce apoptosis in various tumor cells and has been revealed to be relatively non-toxic towards normal cells (7). TRAIL triggers apoptosis by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Activation of these receptors results in a signal transduction cascade that initiates both extrinsic and intrinsic apoptotic pathways (8). TRAIL also binds to two other receptors that lack functional cytoplasmic death domains, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), as well as osteoprotegerin, a secreted TNF receptor homolog. These receptors have been considered to potentially inhibit TRAIL-induced apoptosis. Therefore, use of a specific activator of TRAIL-R1 or TRAIL-R2 would be favorable to exclude potential interference from competition with DcRs. Previous studies have reported that monoclonal antibodies (mAbs) targeting human TRAIL-R1 or TRAIL-R2 from mice

or rabbits exhibit antitumor activities *in vitro* and *in vivo* (9,10). These agonistic antibodies have functions similar to TRAIL in triggering apoptotic pathways mediated by TRAIL activation. In addition, several studies have reported that lexatumumab, a fully human agonistic mAb specific for TRAIL-R2, induces apoptotic cell death in some tumor cells (11-13). It was previously reported by the authors that low concentrations of doxorubicin enhance lexatumumab-mediated apoptosis and cytotoxicity by inducing TRAIL-R2 expression in various human solid cancer cells, including RCC, prostate, bladder and lung cancer cells (14).

Celastrol, a plant triterpene, exhibits a diverse range of pharmacological properties, including anti-inflammatory, antioxidative and antitumor effects (15,16). Previous studies have demonstrated that celastrol has broad-spectrum anticancer activities, including against prostate cancer, glioblastoma, lung cancer, colon cancer, ovarian cancer cells, melanoma, osteosarcoma and RCC (16-23). However, studies have demonstrated that periods of celastrol injection cause obvious weight loss in mice, indicating that its toxicity poses a threat to normal cells and tissues (24,25). Nevertheless, celastrol enhances TRAIL-induced apoptosis in some tumor cells via the death receptor pathway, indicating a potential strategy to effectively reduce associated side effects (25-27).

In the present study, the enhancement of TRAIL-R2-mediated apoptosis and cytotoxicity in RCC cells by celastrol was examined. The molecular mechanisms that are possibly related to enhanced cytotoxicity were also explored.

Materials and methods

Reagents. Celastrol was obtained from Sigma-Aldrich; Merck KGaA, dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted in culture medium. The concentration of DMSO utilized during treatment was <0.1%. Lexatumumab, highly specific for binding to TRAIL-R2, was generously supplied by Human Genome Sciences (28). The concentration of lexatumumab used was 1-100 ng/ml because it did not have significant cytotoxic effects in a variety of cancer cells at 100 ng/ml in previous studies (14,29,30).

RCC cell lines and primary RCC cells. Two human RCC cell lines were used: ACHN (cat. no. CRL-1611) and Caki-1 (cat. no. HTB-46) were obtained from the American Type Culture Collection. Primary RCC cells were separated from the surgical specimens of six patients with untreated RCC as previously described (31). Pathologic stage and grade were consistent with the 2004 worlds health organization (WHO) criteria (https://www.patologi.com/WHO%20kidney%20testis.pdf): T3N0M1 grade 2 in patient 1 (77 years, female), T2N0M0 grade 2 in patient 2 (72 years, male), T3N1M0 grade 2 in patient 3 (68 years, female), T2N0M0 grade 1 in patient 4 (70 years, male), T2N0M0 grade 1 in patient 5 (63 years, male), and T2N0M0 grade 2 in patient 6 (70 years, male). All cells were cultured in RPMI-1640 medium (cat. no. 11875119) supplemented with 10% FBS (cat. no. 10099141) and 1% penicillin and streptomycin (cat. no. 15140122) (all from Gibco; Thermo Fisher Scientific, Inc.), and then maintained at 37˚C in 5% CO_2.

Ethical approval for use of human tissue was granted by Hyogo College of Medicine (approval no. 202306; Hyogo, Japan). All patients provided individual written informed consent for use of their sampled tissues.

Cytotoxicity assays. Cytotoxicity was determined based on colorimetric assays of cell viability using Cell Counting Kit-8 (CCK-8) (cat. no. 343-07623; Dojindo Laboratories, Inc.) (32). Briefly, a 100-μl suspension containing $0.5x10^4$ cells was added to a 96-well flat bottom microtiter plate. Following 24 h of incubation at 37˚C, 100 μl of lexatumumab or celastrol, alone or in combination, or medium alone (control) was added to the plates in triplicate. Each plate was then incubated at 37˚C for an additional 3-24 h. Next, 10 μl of CCK-8 solution was added and incubated for 3 h. Absorbance (A) was quantified using a SPECTRAmax PLUS384 (Molecular Devices, LLC) at 450 nm as the reference, and cell viability was determined based on the percentage of control cells using the following formula: percent cell viability=(*A* of treated wells/*A* of control wells) x100.

The coefficient of drug interaction (CDI) was determined to assess the interactions between celastrol and lexatumumab. CDI was calculated as AB/(AxB), where AB is the *A* of the mixture of the two active agents/*A* of the control, and A and B are the *A* of the single active agent/*A* of the control. Based on the CDI, the interactions were categorized as synergistic (CDI <1), additive (CDI=1), or antagonistic (CDI >1) (33). CDI <0.7 was considered to indicate a significant level of synergism between the drugs.

Cell viability was assessed using a trypan blue dye exclusion test. Initially, cells were seeded in six-well plates at $3x10^5$ cells/well and cultured at 37˚C for 24 h. Subsequently, they were treated in duplicate with lexatumumab and/or celastrol for 6 h. Following treatment, cells were harvested and viable cells counted by staining with a 0.5% trypan blue dye solution (Sigma-Aldrich; KGaA) under an optical microscope (magnification, x10) (Olympus Corporation).

Apoptosis assays. Apoptosis was assessed using two distinct methodologies. First, following incubation with 100 ng/ml of lexatumumab and/or 1 μM celastrol for 6 h, floating and adherent cells were harvested. The obtained cells were washed twice with cold PBS and resuspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. Subsequently, the cells were stained with 5 μl of FITC Annexin V and 5 μl of propidium iodide (PI) (FITC annexin V Apoptosis Detection Kit; cat. no. 556547; BD Biosciences), and then analyzed by an LSRFortessa™ X-20 instrument (BD Biosciences) equipped with the BD FACSDiva and FlowJo (v10.7.1; FlowJo LLC) software packages. Second, DNA fragmentation was quantified using an enzyme-linked immunosorbent assay (ELISA) Kit for cell death detection (cat. no. 11544675001; Roche Life Science Products; Merck KGaA) according to the manufacturer's instructions. Briefly, after treatment with 100 ng/ml of lexatumumab and/or 1 μM of celastrol for 6 h, the cells were resuspended in 500 μl of incubation buffer at a concentration of $1x10^5$ cells/ml at room temperature. After 30 min, the lysate was centrifuged at 20,000 x g for 10 min at 4˚C and the resulting supernatant prediluted 1:10 with incubation buffer. Using a pipette, 100 μl of coating solution was added to each well of MP modules and incubated overnight at 4˚C, after which the incubation buffer was removed and

then 200 μl of incubation buffer were added into each well of the MP modules via pipette and incubated for 30 min at room temperature. After removing the solution, the cells were rinsed three times with washing solution and 100 μl of sample solution added to each well using a pipette and incubated for 90 min at room temperature. Subsequently, the solution was removed and the wells rinsed. A total of 100 μl of conjugate solution (dilution of 10 μl anti-DNA-POD solution with 90 μl incubation buffer) was added to each well using a pipette and incubated for 90 min at room temperature. The solution was then removed and the wells rinsed three times before adding 100 μl of substrate solution to each well using a pipette. The module was placed on a plate shaker and incubated with shaking at 250 x g for 20 min. Finally, the A value of each well was determined using a SPECTRAmax PLUS384 (Molecular Devices, LLC) at 405 nm.

Western blotting. Cells were initially plated in 9-cm plates and held for 24 h. Subsequently, lexatumumab and/or celastrol treatment was performed for 6 h at 37˚C. Following treatment, the cells were lysed for 20 min on ice using lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) and the protein concentrations determined using a Bradford Assay Kit (Bio-Rad Laboratories, Inc.). Next, 30 μg of protein was loaded into each lane of 10% SDS-PAGE gel, and then transferred onto a PVDF membrane after separation. After blocking non-specific binding sites with 5% skim milk in TBS containing 0.1% Tween-20 for 2 h at room temperature, the membranes were incubated overnight at 4˚C with the following primary antibodies: TRAIL-R2 (cat. no. sc-166624), Bid (cat. no. sc-6538), Bcl-2 (cat. no. sc-7382), Bax (cat. no. sc-20067), AIF (cat. no. sc-13116), and caspase-3 (cat. no. sc-7272) obtained from Santa Cruz Biotechnology, Inc. at a 1:100 dilution; FLIP (cat. no. OPA1-01011; https://www.antibodydirectory.com/moreinfos.php?Item=155921) obtained from Thermo Fisher Scientific, Inc. at 1:1,000 dilution; caspase-8 (cat. no. M032-3) and caspase-9 (cat. no. M054-3) purchased from MBL International Co. at a 1:1,000 dilution; and B-actin mouse polyclonal (cat. no. E4D9Z), FADD (cat. no. 2782) and caspase-6 (cat. no. 9762) obtained from Cell Signaling Technology, Inc. at a 1:2,000 dilution. Subsequently, the membranes were washed three times with TBST buffer for 30 min at room temperature, and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (cat. no. 330; MBL International Co.) at a 1:2,000 dilution and HRP-conjugated goat anti-rabbit IgG secondary antibody (sc-2004; Santa Cruz Biotechnology) at a 1:1,000 dilution for 1.5 h at room temperature. Finally, the membranes were washed three times with TBST buffer for 30 min at room temperature. Signals were detected using a chemiluminescence ECL kit (GE Healthcare) and an ImageQuant LAS 4010 system (GE Healthcare).

Caspase activity and inhibition assays. To assess the activities of caspase-8, -9, -6, and -3, quantitative colorimetric assays were performed with caspase-specific kits (BioVision, Inc.) (34). Briefly, cells were treated with 100 ng/ml lexatumumab and/or 1 μM celastrol in a cell culture incubator at 37˚C for 3-24 h. Following treatment, cells were homogenized in 200 μl of cell lysis buffer, and then incubated on ice for 10 min. Next, the lysate was centrifuged at 10,000 x g for 1 min at 4˚C and the supernatant collected to determine the protein concentrations. For the assay procedure, 50 μl of cell lysate with 100 μg of total protein, 50 μl of 2X reaction buffer, and 5 μl of a 4 mM Asp-Glu-Val-Asp-*p*NA (cat. no. K113-100), Val-Glu-lle-Asp-*p*NA (cat. no. K119-100), lle-Glu-Thr-Asp-*p*NA (cat. no. K115-100), or Leu-Glu-His-Asp-*p*NA (cat. no. K106-100) substrate (all from BioVision, Inc.) were added to each well of a 96-well plate and incubated at 37˚C overnight. The A value of each well was determined using a SPECTRAmax PLUS384 (Molecular Devices, LLC) at 405 nm.

Caspase inhibition assays were performed with the caspase-8 inhibitor Z-LETD-FMK (cat. no. C8734), caspase-9 inhibitor Z-LEHD-FMK (cat. no. C1355), caspase-6 inhibitor Z-VEID-FMK (cat. no. C1730), caspase-3 inhibitor Z-DQMD-FMK (cat. no. C0480) (all from Sigma-Aldrich; Merck KGaA), or the general caspase inhibitor Z-VAD-FMK (cat. no. HY-16658Bp; MedChemExpress), or the human recombinant DR5:Fc chimeric protein (cat. no. ALX-522-005-C050; Enzo Life Sciences, Inc.). Cells were pre-treated with the respective caspase inhibitor (50 μM) or DR5:Fc chimeric protein (1 μg/ml) for 1 h, and then exposed to 1 μM celastrol and 100 ng/ml lexatumumab for 6 h. Cell viability was assessed using a CCK-8 kit.

Statistical analysis. Each experiment was performed at least three times, and the results are presented as the mean ± SD. The data were analyzed using one-way analysis of variance (ANOVA). In addition, homogeneity of variance was tested, and post hoc multiple comparisons tests were performed. Data with equal variances were compared using Tukey's post hoc multiple comparisons test and data with unequal variances were compared with Dunnett's T3 test. Two-way ANOVA test was performed for the drug combination assays. GraphPad Prism V8 for MacOS (Dotmatics) was used for all of the analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Celastrol enhances lexatumumab-induced cytotoxicity in RCC cells. First, the effects of different concentrations of celastrol on cell viability were examined using the human ACHN RCC cell line. After a 24 h treatment period, celastrol suppressed cell proliferation in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) of 1.099 μM. Similar cytotoxic effects were noted in examinations of another RCC cell line, Caki-1, as well as primary RCC cells obtained from 6 patients (Fig. 1).

Next, the enhancement of lexatumumab-induced cytotoxicity in RCC cells by celastrol was assessed. A significant potentiation of cytotoxicity was achieved in ACHN cells 24 h after treatment with a combination of lexatumumab and celastrol (Fig. 2A). Such an enhanced cytotoxic effect was also observed when the lexatumumab and celastrol treatment duration was shortened from 24 to 6 h, though there was no effect when shortened further to 3 h (Fig. 2B). Treatment with the combination of lexatumumab and celastrol led to a significant decrease in the number of ACHN cells in trypan blue dye exclusion tests,

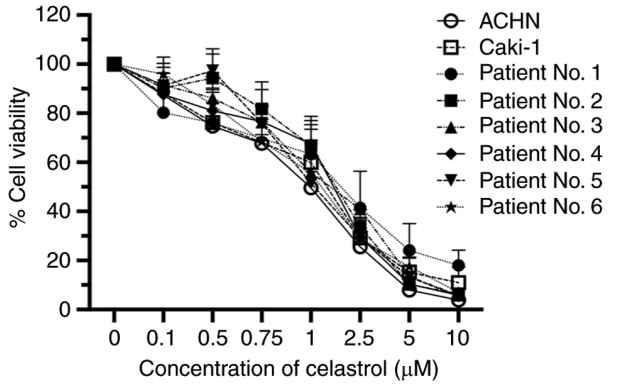

Figure 1. Dose-dependent cytotoxicity of celastrol in RCC cells. ACHN, Caki-1 and 6 patients with primary RCC cell types were treated with 0.1-10 μM celastrol for 24 h. Cell viability was determined using a Cell Counting Kit-8 kit. Values are shown as the mean ± SD of three individual experiments. RCC, renal cell carcinoma.

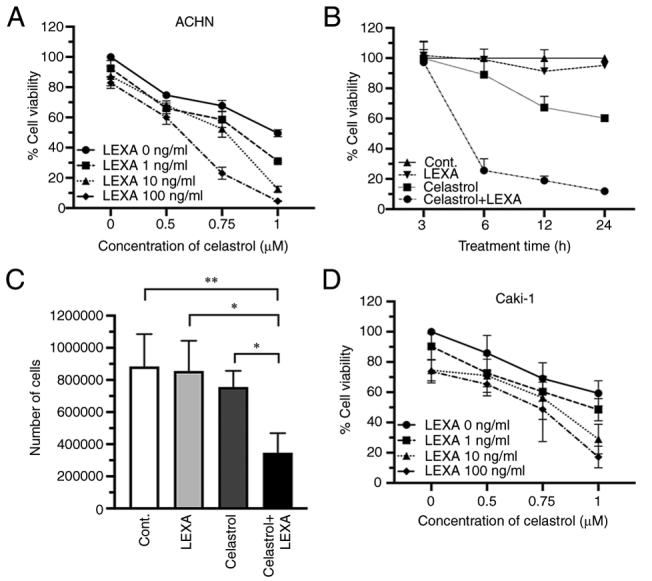

Figure 2. Enhanced cytotoxicity of lexatumumab and celastrol in ACHN and Caki-1 cells. (A) ACHN cells were treated with lexatumumab (1-100 ng/ml) alone, celastrol (0.5-1 μM) alone, or the combination for 24 h. (B) ACHN cells were treated with 100 ng/ml lexatumumab alone, 1 μM celastrol alone, or the combination for 3 h to 24 h. (C) ACHN cells were treated with 100 ng/ml lexatumumab and/or 1 μM celastrol for 6 h. Cell viability was determined using a Cell Counting Kit-8 kit and cell number by trypan blue dye exclusion. (D) Caki-1 cells were treated with lexatumumab (1-100 ng/ml) alone, celastrol (0.5-1 μM) alone, or the combination for 24 h. LEXA, lexatumumab; Cont., control. Values are shown as the mean ± SD of three individual experiments. *P<0.05 and **P<0.01 vs. untreated control.

whereas treatment with either alone led to only a slight decrease (Fig. 2C). A similar enhanced cytotoxic effect of lexatumumab and celastrol was observed in Caki-1 cells (Fig. 2D).

The cytotoxic effects of lexatumumab and celastrol were also evaluated in primary RCC cells obtained from 6 patients. In each case, significant enhancement of cytotoxic effects and synergy was identified, regardless of the sensitivity of RCC cells to celastrol or lexatumumab when each was used alone (Table I and Fig. 3A-F).

Taken together, these findings clearly demonstrated that treatment with lexatumumab and celastrol in combination

Table I. The CDI.

	Lexatumumab (100 ng/ml)	
Renal cell carcinoma cells	Celastrol 0.5 μM	Celastrol 1 μM
ACHN	0.96825323	0.112518579
Caki-1	0.76507638	0.391270559
Patient No. 1	1.05027777	0.443204229
Patient No. 2	0.995838373	0.394370361
Patient No. 3	0.921209366	0.537397991
Patient No. 4	1.249032099	0.394213415
Patient No. 5	0.916766395	0.287956402
Patient No. 6	0.974511663	0.522562291

The CDI value of less than, equal to or greater than 1 indicates that the drugs are synergistic, additive, or antagonistic, respectively. A CDI value below 0.7 signifies synergism between the drugs. CDI, coefficient of drug interaction.

enhances cytotoxicity towards RCC cell lines and primary RCC cells.

Induction of apoptosis. RCC cells were analyzed to improve evaluation of whether the observed enhancement of cytotoxicity was mediated by apoptosis. Enhanced apoptosis of cells treated with lexatumumab in combination with celastrol was detected by both flow cytometry (Fig. 4A and B), and a quantitative apoptosis-specific ELISA kit (Fig. 4C). Thus, the enhanced cytotoxicity of lexatumumab and celastrol was related to their ability to trigger apoptotic cell death.

Synergistic cytotoxicity of lexatumumab and celastrol is TRAIL-R2-dependent. Western blot analysis was performed to determine whether expression of TRAIL-R2 was related to the sensitization of RCC cells to lexatumumab-induced apoptosis by celastrol. Celastrol remarkably increased TRAIL-R2 expression in RCC cells in both a dose- and time-dependent manner (Fig. 5A and B).

To further assess the underlying molecular mechanism of enhanced cytotoxicity induced by the combination of lexatumumab and celastrol, the authors examined the effects of a human recombinant DR5:Fc chimeric protein (cat. no. ALX-522-005-C050; Enzo Life Sciences, Inc.) known to have a dominant role negating the cytotoxicity of TRAIL-R2. As demonstrated in Fig. 5C, celastrol-mediated enhancement of lexatumumab-induced cytotoxicity was significantly inhibited when the DR5:Fc chimeric protein was present.

These results indicated a TRAIL-R2-dependency of the synergistic cytotoxicity and apoptosis that occurs with the combination of lexatumumab and celastrol.

Effects of lexatumumab and celastrol combination on expression of apoptotic compounds. The regulation of compounds related to TRAIL-R2-mediated apoptosis were examined by the combination of lexatumumab and celastrol in

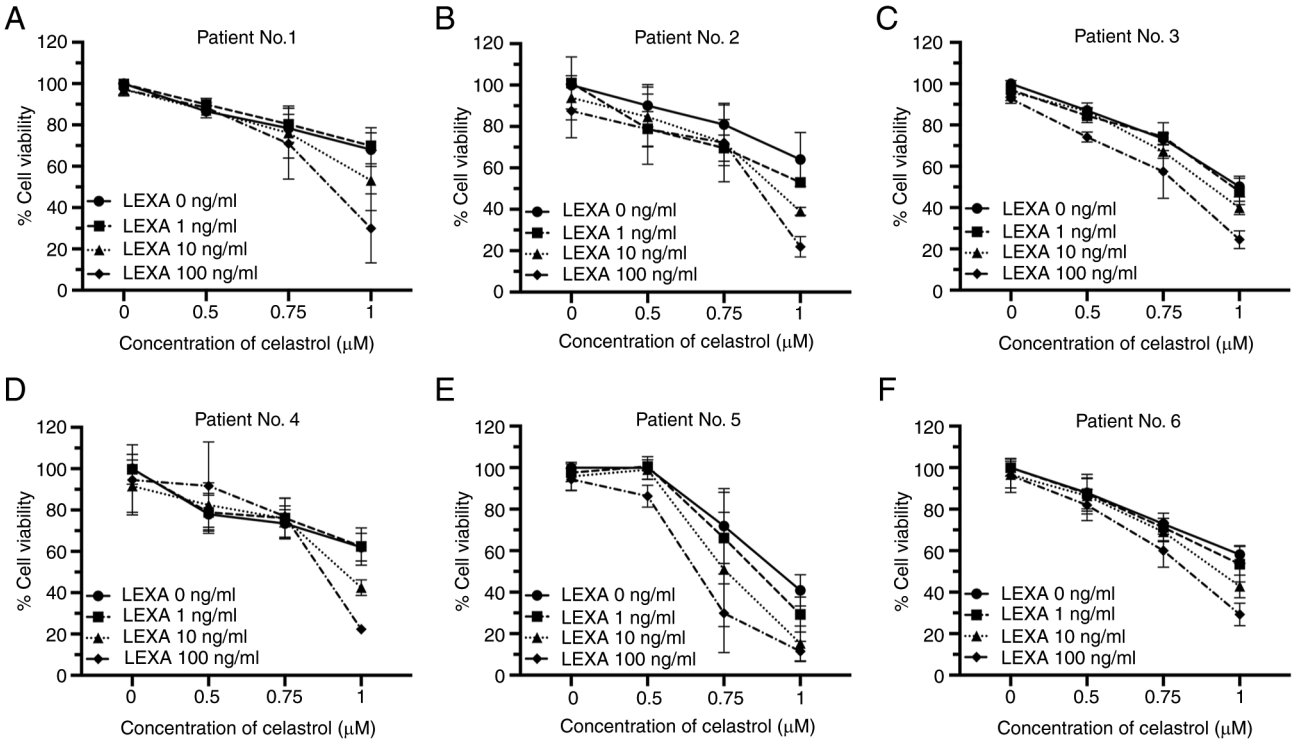

Figure 3. Enhanced cytotoxicity of lexatumumab and celastrol in primary RCC cells. (A-F) Primary RCC cells derived from 6 patients were treated with lexatumumab (1-100 ng/ml) alone, celastrol (0.5-1 μM) alone, or the combination for 24 h. Cell viability was determined using a Cell Counting Kit-8 kit. Values are shown as the mean ± SD of three individual experiments. RCC, renal cell carcinoma; LEXA, lexatumumab.

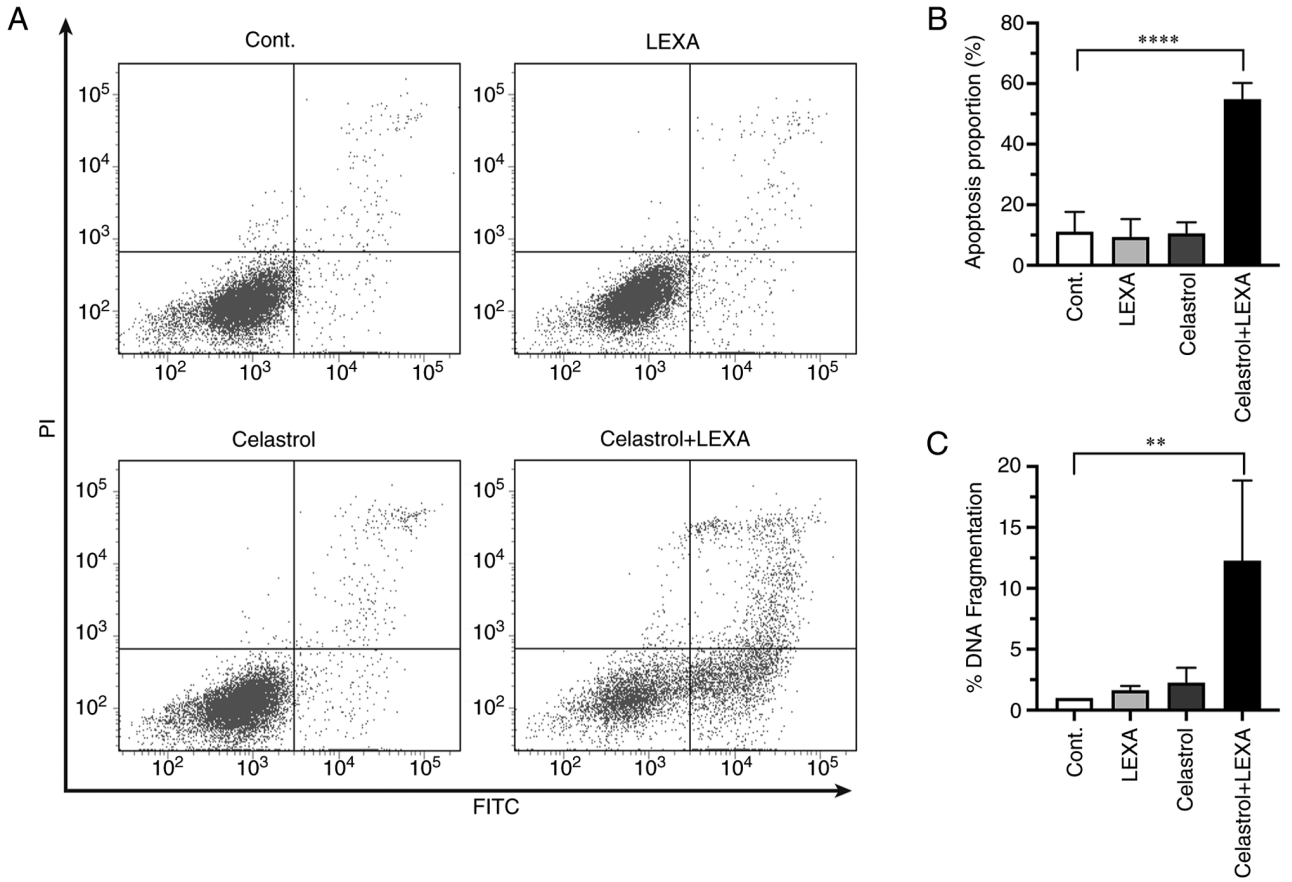

Figure 4. Synergistic apoptosis induced by lexatumumab and celastrol. (A and B) Flow cytometric analysis of apoptotic cells. ACHN cells were treated for 6 h with 100 ng/ml lexatumumab and/or 1 μM celastrol, and then stained with Annexin V-FITC and PI. Cells positive for Annexin V-FITC and PI were assessed by flow cytometry. (C) DNA fragmentation was quantified based on apoptosis-specific ELISA findings. Values are shown as the mean ± SD of three individual experiments. **P<0.01 and ****P<0.0001 vs. untreated control. LEXA, lexatumumab; Cont., control.

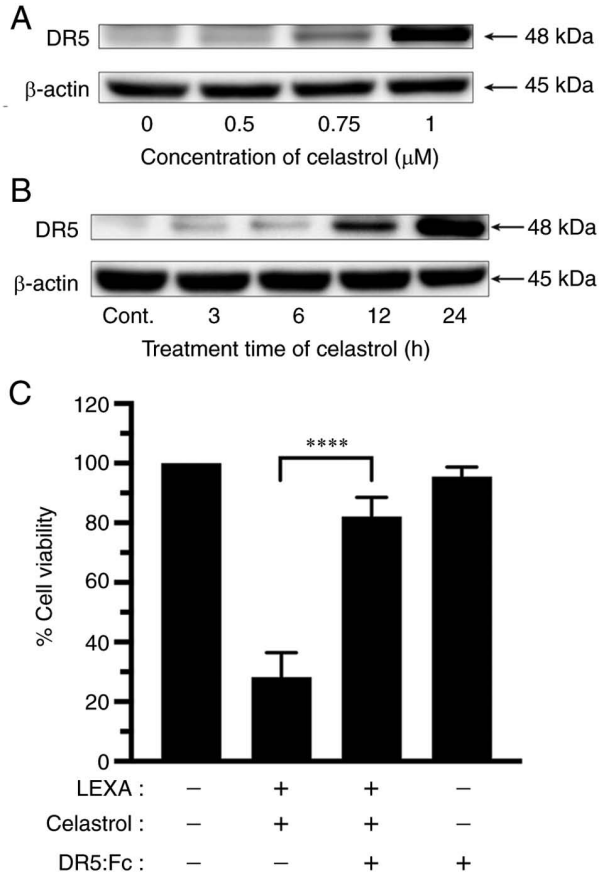

Figure 5. TRAIL-R2-dependent synergistic cytotoxicity of lexatumumab and celastrol. (A) ACHN cells were treated with 0.5-1 μM celastrol for 24 h or (B) 1 μM celastrol for 3 to 24 h. TRAIL-R2 expression was determined by western blot analysis. β-actin was used as a loading control. (C) Cells were treated with 100 ng/ml lexatumumab and 1 μM celastrol in the absence or presence of 1 μg/ml DR5:Fc for 6 h. Cell viability was determined using a Cell Counting Kit-8 kit. Values are shown as the mean ± SD of three individual experiments. ****P<0.0001 vs. untreated control. LEXA, lexatumumab; Cont., control.

RCC cells using western blotting. Expression of FADD, FLIP, Bid, Bax, Bcl-2 and AIF was not affected when RCC cells were treated with celastrol and/or lexatumumab for 6 h. However, the combination treatment remarkably induced activation of cleaved caspase-8, -9, -6, and -3 (Fig. 6A).

Activation of caspase cascade by lexatumumab and celastrol. Minor activation of caspase-6 and -3 in RCC cells was observed following lexatumumab treatment, but with no detectable activation of caspase-8 or -9. Exposure to celastrol alone did not activate caspase-8 or -9, whereas activation of caspase-6 and -3 was slightly induced, though their levels were significantly lower than in treatment with the combination of lexatumumab and celastrol. The combination induced remarkable activation of caspase-8, -9, -6, and -3 (Fig. 6B).

Suppressive effects of VAD and DR5:Fc chimeric protein toward caspase activation. The activities of caspase-8, -9, -6 and -3 in cells treated with lexatumumab in combination with celastrol in the absence or presence of the general caspase inhibitor or DR5:Fc chimeric protein were also examined. Caspase-8, -9, -6 and -3 activities in the cells, which were elevated by use of the drug combination, were significantly suppressed by both Z-VAD-FMK (Fig. 7A) and DR5:Fc chimeric protein (Fig. 7B).

Caspase inhibitors inhibit synergistic cytotoxic effect of lexatumumab and celastrol. To confirm mediation of the synergistic cytotoxic effect of lexatumumab and celastrol through caspase activation, the effects of specific inhibitors of caspase-8, -9, -6 and -3, as well as a general caspase inhibitor, on cell death induced by lexatumumab and celastrol, were examined. The findings showed that cytotoxicity was significantly inhibited by the specific inhibitors of caspase-8, -9, -6, and -3, as well as the general caspase inhibitor (Fig. 8).

Discussion

From a clinical perspective, combination treatment with lexatumumab and celastrol is promising. The present study indicated that celastrol at a low concentration (0.5-1 μM) in combination with lexatumumab exerts a synergistic effect not only on human RCC cell lines, but also primary RCC cells derived from patients. It was also revealed that TRAIL-R2-dependent induction of apoptosis and caspase cascade activation, indicate synergistic cytotoxicity of celastrol and lexatumumab. Interestingly, the growth of RCC cells was significantly inhibited at 24 h by treatment with 1 μM celastrol plus 100 ng/ml lexatumumab. A similar result was achieved when the treatment duration was shortened to 6 h. These findings suggested that the synergistic cytotoxicity of lexatumumab and celastrol is due to the induction of apoptosis via upregulation of TRAIL-R2 expression and activation of the caspase cascade.

Cell surface expression of TRAIL-R1 or TRAIL-R2 plays a crucial role in mediating TRAIL-induced apoptosis, though the sensitivity of tumor cells expressing these death receptors to TRAIL is not always consistent due to intracellular mechanisms (35). Furthermore, the efficacy of TRAIL correlated with levels of TRAIL-R1 and/or TRAIL-R2 on the surface of leukemia cells (36). On the other hand, previous studies have found no connection between TRAIL receptor expression and the synergy of TRAIL and chemotherapeutic drugs in examinations of certain cell lines (37,38). The results of the present study demonstrated that celastrol significantly upregulated TRAIL-R2 expression in RCC cells. The synergistic cytotoxicity of lexatumumab and celastrol was also significantly inhibited by DR5:Fc chimeric protein, which had a dominant negative function against TRAIL-R2.The findings of the present study suggested that lexatumumab and celastrol induce cytotoxicity and apoptosis in RCC cells synergistically through upregulation of TRAIL-R2.

Caspases are essential protease mediators of apoptosis known to be triggered by various stimuli, including TRAIL (39,40), but it is difficult to examine isolated TRAIL-mediated signal transduction because some receptors complicate this transduction. Using lexatumumab, a specific TRAIL-R2 mAb, the authors were able to specifically assess caspase involvement in TRAIL-R2-mediated apoptosis. The findings of the present study demonstrated that lexatumumab and celastrol in combination significantly activate initiative caspases, such as caspase-8 and -9, and effective caspases, including caspase-6

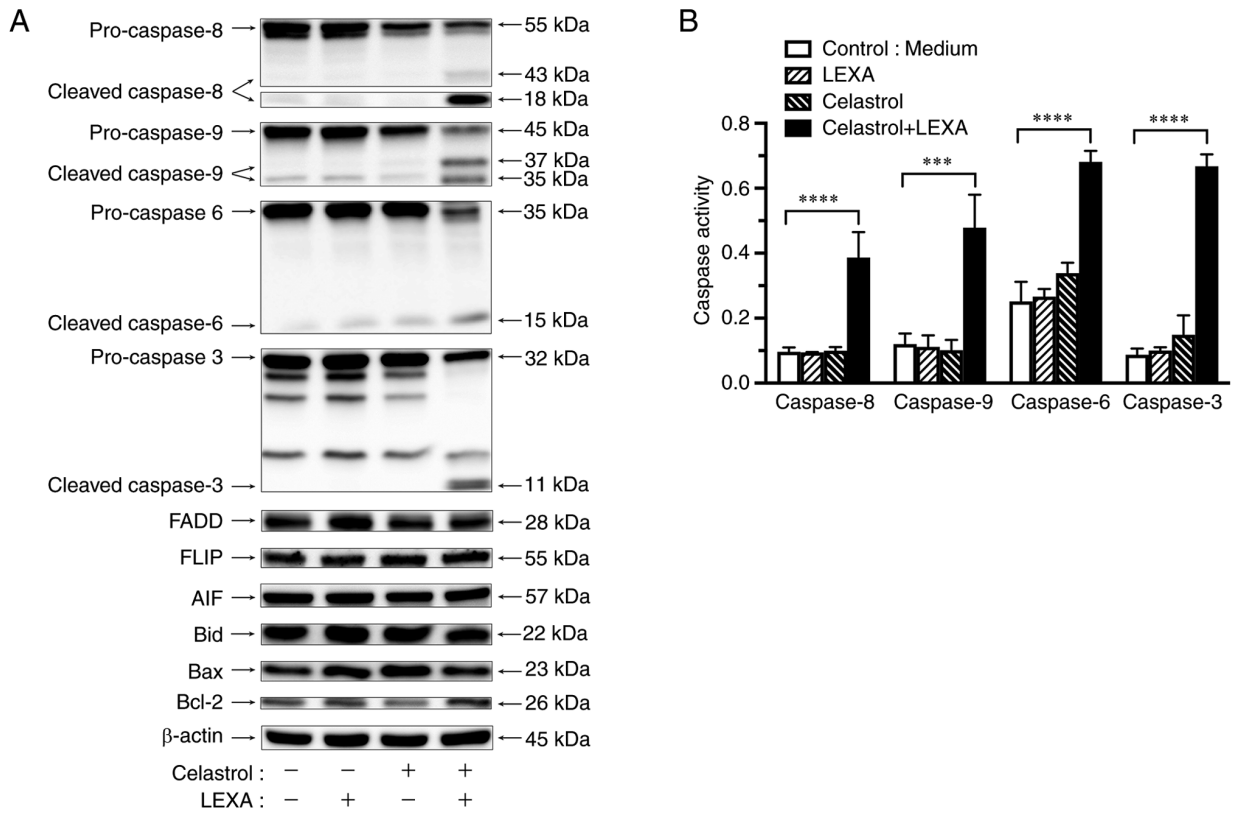

Figure 6. Activation of caspases by lexatumumab and celastrol. ACHN cells were treated with 100 ng/ml lexatumumab alone, 1 μM celastrol alone, or the combination for 6 h. (A) Expression of caspase-8, -9, -6, and -3, AIF, Bid, Bax and Bcl-2 was determined by western blot analysis. β-actin was used as a loading control. (B) Activities of caspase-8, -9, -6 and -3 were determined by colorimetric assay. Values are shown as the mean ± SD of three individual experiments. ***P<0.001 and ****P<0.0001 vs. untreated control. LEXA, lexatumumab; Cont., control.

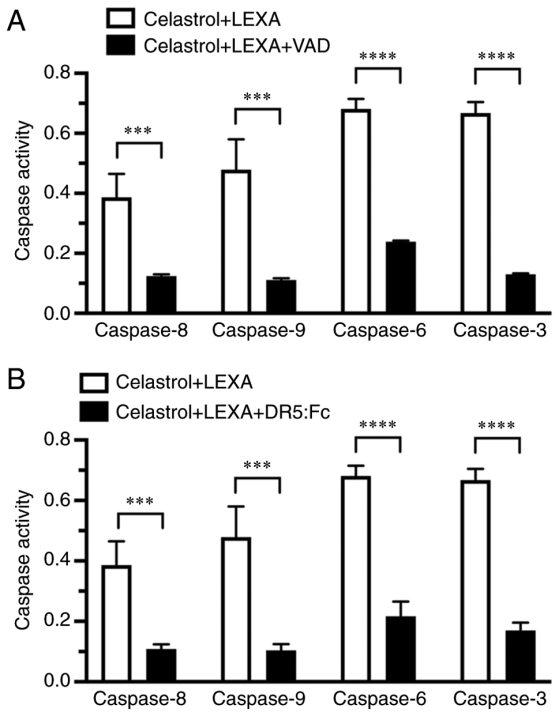

Figure 7. Suppressive effects of VAD and DR5:Fc chimeric protein on the activation of caspases. ACHN cells were treated with 100 ng/ml lexatumumab and 1 μM celastrol for 6 h in the absence or presence of (A) 50 μM VAD or (B) 1 μg/ml DR5:Fc chimeric protein. The activities of caspase-8, -9, -6 and -3 were determined by colorimetric assay. Values are shown as the mean ± SD of three individual experiments. ***P<0.001 and ****P<0.0001 vs. untreated control. LEXA, lexatumumab; Cont., control.

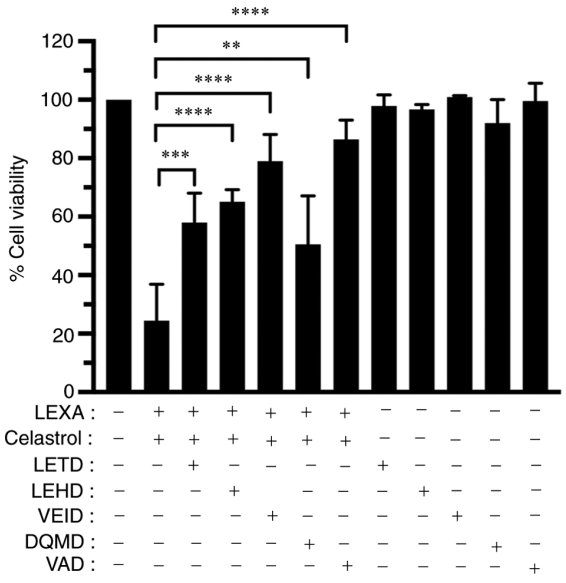

Figure 8. Caspase inhibitors block the synergistic cytotoxicity of lexatumumab and celastrol. ACHN cells were treated with 100 ng/ml lexatumumab and 1 μM celastrol for 6 h in the absence or presence of 50 μM LETD, LEHD, VEID, DMQD, or VAD. Cell viability was determined using a Cell Counting Kit-8 kit. Values are shown as the mean ± SD of three individual experiments. **P<0.01, ***P<0.001 and ****P<0.0001 vs. untreated control. LEXA, lexatumumab.

and -3. The elevated activities of caspase-8, -9, -6 and -3 in cells exposed to the drug combination were significantly suppressed

by the DR5:Fc chimeric protein and general caspase inhibitor Z-VAD-FMK, whereas the synergistic cytotoxicity of lexatumumab and celastrol was inhibited considerably by specific inhibitors of caspase-8, -9, -6 and -3, as well as a general caspase inhibitor. Taken together, these findings indicated that the caspase cascade comprised molecules downstream of death receptors plays a crucial role in the synergistic cytotoxicity of lexatumumab and celastrol in RCC cells.

A previous study demonstrated that celastrol enhances TRAIL-mediated apoptosis through upregulation of TRAIL-R2 and the activation of caspase-8 and -3 in human glioblastoma cells, whereas celastrol has no effect on TRAIL-mediated apoptosis in normal human astroglial cells (27). Another study found that simultaneous administration of TRAIL and celastrol had an anticancer effect against human colorectal tumors in nude mice (26). The results of the present study indicated that lexatumumab and celastrol have a synergistic effect not only on human RCC cell lines, but also primary RCC cells. Thus, treatment of RCC with the combination of celastrol and lexatumumab is promising for potential clinical application.

The toxicity of celastrol exposure to normal kidney cells is a concern. Consequently, a method to alleviate the toxicity of celastrol is needed. Some studies have demonstrated that low concentrations of celastrol enhance TRAIL-induced apoptosis in some tumor cells via the death receptor pathway, indicating a potential strategy to effectively reduce associated side effects (25-27). In the present study, the authors focused on how to reduce the cytotoxicity of celastrol through combination with lexatumumab in RCC cells. Further studies will be needed to clarify the cytotoxic effect of celastrol in combination with lexatumumab on normal kidney cells.

In conclusion, the results obtained in the present study clearly indicated that administration of lexatumumab with celastrol has cytotoxic effects on both human RCC cell lines and primary RCC cells, particularly at a low concentration of celastrol (0.5-1 μM). This synergistic cytotoxicity of lexatumumab and celastrol is due to induction of apoptosis by the upregulation of TRAIL-R2 expression and activation of the caspase cascade. Thus, the use of celastrol in combination with lexatumumab may be a novel therapeutic strategy for treatment of RCC.

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Availability of data and materials

All data generated or analyzed during this study are included in the current published article.

Authors' contributions

YB, YKi and YKa performed the cell proliferation and western blot assays, and statistical analysis of the data obtained in all of the experiments.TK and AK performed the trypan blue staining analysis. SY and XW planned, analyzed and interpreted all of the experiments and validity of the data, and drafted the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work and data are appropriately investigated and resolved. YB and XW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical approval for the use of human tissue was granted by Hyogo College of Medicine (approval no. 202306; Hyogo, Japan). All patients provided individual written informed consent for the use of their sampled tissues.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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