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ORIGINAL

Induction of apoptotic cell death by calcitriol in RAW264.7 cells

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ABSTRACT : Vitamin D affects bone and mineral metabolism and immunity. Epidemiological data suggests that vitamin D may play a role in the development and progression of cancer. Additionally, calcitriol, an active form of vitamin D, has an antiproliferative effect on malignant tumors. However, it has also been reported that calcitriol suppresses cell death, suggesting that the effects of calcitriol may vary depending on the type of cell. In this study, we aimed to reveal the effects of calcitriol stimulation on macrophage-like cells derived from tumors induced by murine leukemia virus (RAW264.7 cells) and the cell death pathway.

RAW264.7 cells were stimulated with calcitriol. Cell death was determined by measuring the lactate dehydrogenase (LDH) levels in the supernatant and the percentage of cells stained with annexin V/propidium iodide (PI). Cell death pathways were examined using Western blotting.

LDH levels were increased in concentration- and time-dependent manners after calcitriol stimulation. Annexin V/PI staining showed that calcitriol stimulation increased the percentage of cells stained with both annexin V and PI compared with that of the control. Calcitriol stimulation increased the expression of cleaved caspase-3 in the supernatants. A necroptosis inhibitor, Necrostatin-1, did not affect the level of LDH induced by calcitriol stimulation.

Calcitriol induces cell death (apoptosis; not necroptosis) in RAW264.7 cells.

Key Words : RAW264.7, calcitriol, vitamin D, apoptosis

Introduction

Humans obtain vitamin D from diets and supplements or synthesize it by sunbathing[1–3]. In the body, vitamin D is metabolized to its active form, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] or calcitriol. Calcitriol binds to the vitamin D receptor in the nucleus, and forms heterodimers with the retinoid X receptor and its ligand (9 *cis*-retinoic acid). These dimers occupy specific nucleotide sequences (i.e., vitamin D response elements). In conjunction with other transcription factors, this complex exerts physiological functions in target tissues by inducing and adjusting the transcription of vitamin D-responsive genes[1,4,5]. $1,25(OH)_2D_3$ regulates calcium and phosphate homeostasis by affecting the small intestine, kidneys, and bones[1]. In addition, calcitriol regulates several signaling pathways, including inflammation, differentiation, apoptosis, proliferation, cell cycle, and angiogenesis[4,5]. Therefore, calcitriol may affect immunity and cancer.

In the 1980s, calcitriol was confirmed to induce the differentiation of human myeloid leukemia or HL-60 cells[6]. Calcitriol has also been found to suppress proliferation

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and increase phagocytosis in HL-60 cells[7, 8]. Furthermore, calcitriol can reduce the proliferation and induce the differentiation of other leukemia cells[9–12]. These results suggested that calcitriol suppresses cancer progression. Several studies have reported that calcitriol induces anti-proliferation, anti-angiogenesis, cell cycle arrest, apoptosis, and autophagy in other cancers, including colon cancer[13], breast cancer[14, 15], and melanoma[16].

In contrast, other studies have shown that calcitriol prevents apoptosis in several leukemia cell lines[17, 18]. We believe that calcitriol has various effects depending on the cell type. However, its effects and mechanisms of action have not been clearly elucidated.

In this study, we focused on the effects of calcitriol stimulation on macrophage-like cells derived from tumors induced by murine leukemia virus (RAW264.7 cells) and the cell death pathway. This is a pilot study investigating the effects of calcitriol on cancer cells by analyzing the effects on RAW264.7 cells.

Materials and methods

Cell line

Macrophage-like cells derived from tumors induced by murine leukemia virus (RAW264.7, ATCC TIB-71) were from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in Roswell Park Memorial Institute (RPMI)1640 medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin. RAW264.7 cells were seeded in 24-well plates at a density of 5×10^5 cells/well (lactate dehydrogenase [LDH] assay) or six-well plates at a density of 2×10^6 cells/well (annexin V/propidium iodide [PI] staining, immunoblotting). Cells were maintained in a 37 °C incubator containing 5% CO_2.

Calcitriol and pan-caspase inhibitor

Calcitriol (FUJIFILM, Tokyo, Japan) was dissolved in ethanol and stored at -80°C. Pan-caspase inhibitor Z-VAD -FMK (PEPTIDE INSTITUTE. INC., Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C.

Observation of cell shape

RAW264.7 cells were treated with or without Z-VAD-FMK (50 μM) for 2 h and stimulated with calcitriol (10 μM) for 3 h. Cells were observed by using a phase contrast microscope at a ×10 magnification. Images of the cells were captured with a DIGITAL MICROSCOPE IMAGER (CELESTRON, Torrance, CA, USA) and S-Viewer V1.18.7.27 (CELESTRON).

LDH assay

RAW264.7 cells were treated with or without Z-VAD-FMK (50 μM) for 2 h and stimulated with calcitriol (10 μM) for 3 h. The LDH levels in the supernatants was measured using the Cytotoxicity LDH Assay Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Annexin V/PI staining

For analyzing cell death, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and PI and analyzed by using NovoCyte® Flow Cytometer (Agilent Technologies, Santa Clara, CA, USA). RAW264.7 cells were treated with or without Z-VAD-FMK (50 μM) for 2 h and stimulated with calcitriol (10 μM) for 3 h. Cells were subsequently trypsinized and washed with PBS. The stimulated cells were stained using a MEBCYTO-apoptosis Kit (MEDICAL & BIOLOGICAL LABORATORIES, Tokyo, Japan) according to the manufacturer's protocol. After staining, the cells were counted using a NovoCyte® Flow Cytometer and data were processed using the NovoExpress Software Ver. 1.5.0 (Agilent Technologies).

Immunoblotting

RAW264.7 cells were treated with or without Z-VAD-FMK (50 μM) for 2 h and stimulated with calcitriol (10 μM) for 3 h. The cells were collected using a scraper and washed with PBS. Then the cells were lysed in RIPA Buffer (Cell Signaling Technology, Danvers, MA, USA) containing phenylmethylsulfonyl fluoride and the concentration of protein in the cell lysates were measured using Quick Start™ Bradford 1 × Dye Reagent (Bio-RAD, Hercules, CA, USA) by Bradford assay. The proteins in the lysates were transferred to a PVDF membrane using Trans-Blot Turbo (BIO-RAD) and reacted with anti-cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb (Cell Signaling; 1:1000), anti-caspase-3 (D3R6Y) Rabbit mAb (Cell Signaling; 1:1000), or anti-β actin (AC-15, Sigma-Aldrich; 1:10000).

Necroptosis inhibitor

Necroptosis inhibitor, Necrostatin-1 (MedChemExpress, South Brunswick, NJ, USA), was dissolved in DMSO and stored at -20°C. RAW264.7 cells were treated with Necrostatin-1 (12.5, 25, 50, and 100 μM) for 2 h and

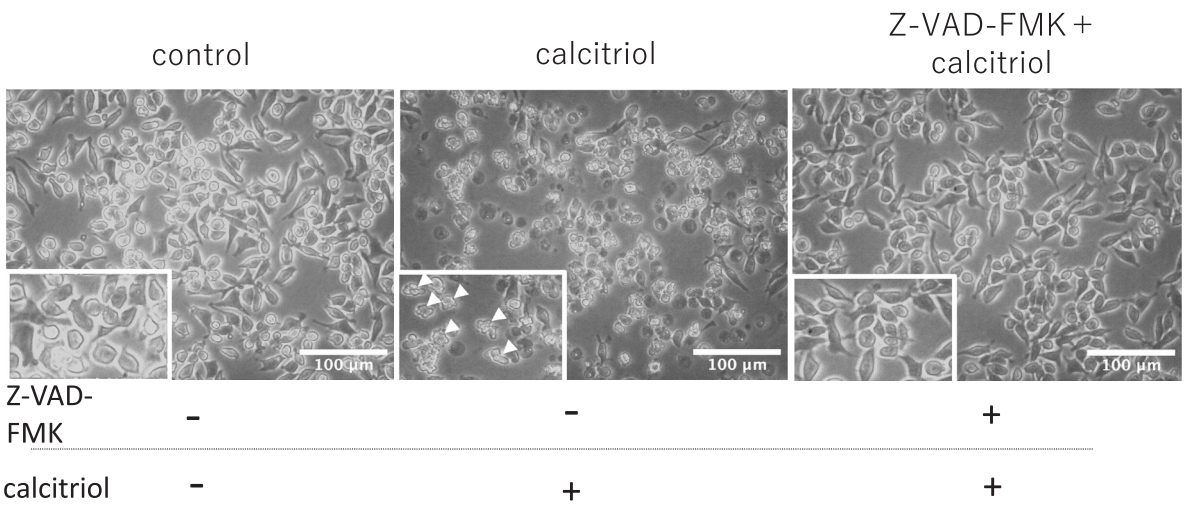

Fig. 1. The effects of calcitriol and Z-VAD-FMK on RAW264.7 cell morphology. The cells were pretreated with or without Z-VAD-FMK (50 μM) for 2 h and treated with calcitriol (10 μM) for 3 h. DMSO and ethanol were used as controls. The image in the white box in the lower left shows an enlarged image. The white arrows indicate cells whose shape has changed due to stimulation with calcitriol. Representative images were obtained using a phase-contrast microscope at 10-fold magnification.

stimulated with calcitriol (10 μM) for 3 h. The LDH content in the supernatant was measured as described above.

Statistical analysis

Data are presented as mean ± S.D. Statistical analyses were performed using Tukey's honest significant difference test using R software (version 4. 2. 2; The R Foundation, 2024). Values with $p < 0.05$ were considered significantly different.

Results

Calcitriol changed the shape of RAW264.7 cells

The shape of RAW264.7 cells was observed under a phase-contrast microscope at 10× magnification. Cells stimulated with calcitriol showed morphological alterations compared with that of control cells (Fig. 1). Calcitriol stimulation changed the shape of the cells from round or oval to random. The shape changes in the cells might have indicated that cell death was induced by calcitriol; thus, the effect of Z-VAD-FMK on these changes was examined.

As expected, Z-VAD-FMK pre-treatment of RAW264.7 cells attenuated the induction of the changes in cell shape, suggesting that caspase activity is involved in calcitriol-induced cell death.

Calcitriol stimulation increased the LDH levels in RAW264.7 culture supernatants

Following calcitriol stimulation, the LDH levels in RAW264.7 culture supernatants were examined. Calcitriol stimulation increased the LDH levels in a concentration-dependent manner. There were significant differences in the LDH levels induced with 10 and 50 μM of calcitriol compared with that of the control (Fig. 2a). In addition, calcitriol (10 μM) stimulation increased the LDH levels in a time-dependent manner. There were significant differences in the LDH levels after 3, 6, 9, and 12 h of stimulation with calcitriol compared with that in the control (Fig. 2b). Moreover, the increase in LDH induced by calcitriol (10 μM) stimulation was significantly reduced by Z-VAD-FMK (50 μM) pretreatment (Fig. 2c).

These results showed that calcitriol induced the death of RAW264.7, which was suppressed by pre-treatment with Z-VAD-FMK. Therefore, calcitriol stimulation induces cell death via caspase activation.

Calcitriol stimulation induced early apoptosis and necrosis

Calcitriol-stimulated cells were stained with annexin V and PI, and analyzed using flow cytometry. Annexin V stains apoptotic cells. PI stains the nucleus but is impermeable to cell membranes. Early apoptotic cells have intact cell membranes, so these cells are not stained with PI. On the other hand, the structures of the cell membranes of late apoptotic cells and necrotic cells are broken, thus the nuclei are stained with PI.

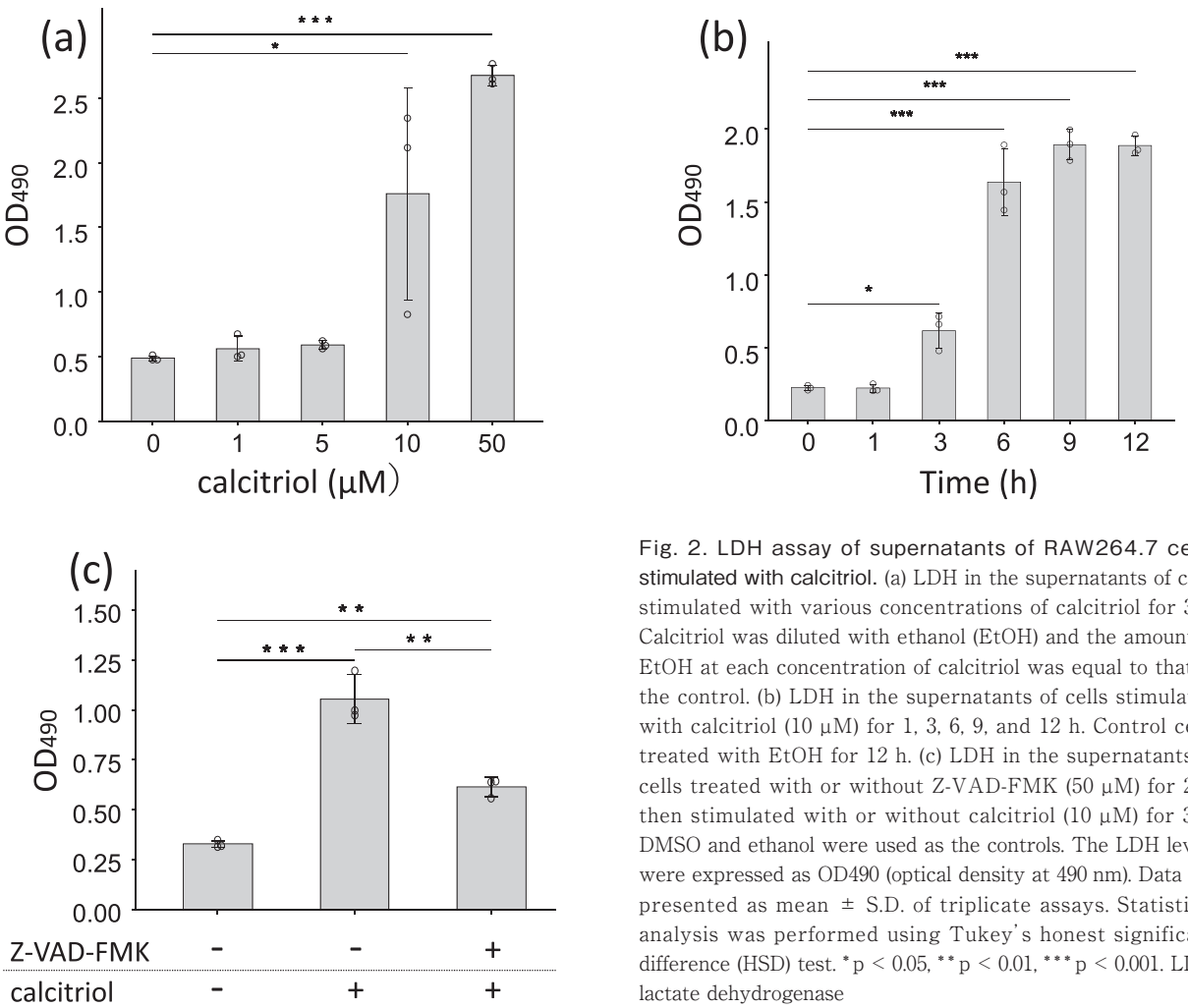

Fig. 2. LDH assay of supernatants of RAW264.7 cells stimulated with calcitriol. (a) LDH in the supernatants of cells stimulated with various concentrations of calcitriol for 3 h. Calcitriol was diluted with ethanol (EtOH) and the amount of EtOH at each concentration of calcitriol was equal to that of the control. (b) LDH in the supernatants of cells stimulated with calcitriol (10 μM) for 1, 3, 6, 9, and 12 h. Control cells treated with EtOH for 12 h. (c) LDH in the supernatants of cells treated with or without Z-VAD-FMK (50 μM) for 2 h, then stimulated with or without calcitriol (10 μM) for 3 h. DMSO and ethanol were used as the controls. The LDH levels were expressed as OD490 (optical density at 490 nm). Data are presented as mean ± S.D. of triplicate assays. Statistical analysis was performed using Tukey's honest significant difference (HSD) test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. LDH, lactate dehydrogenase

Calcitriol stimulation increased the percentage of both early apoptotic and necrotic cells compared with that of the control. Additionally, RAW264.7 cells pretreated with Z-VAD-FMK and stimulated with calcitriol showed an increase in living cells and a decrease in early apoptotic cells compared with those of the cells stimulated with calcitriol alone (Fig. 3a).

The percentage of early apoptotic cells were significantly increased in cells stimulated with calcitriol compared with that of the control. In addition, pre-treatment with Z-VAD-FMK suppressed the increase in the percentage of early apoptotic cells (Fig. 3b).

The percentage of necrotic cells was also significantly increased in cells stimulated with calcitriol compared with that of the control. However, pre-treatment with Z-VAD-FMK did not significantly affect the percentage of necrotic cells (Fig. 3c).

Therefore, it was suggested that caspase-related cell death, which is speculated to be mainly due to apoptosis, is involved in calcitriol-induced cell death in RAW 264.7 cells.

Calcitriol stimulation changed the expression of caspase in RAW264.7 cells

Calcitriol stimulation increased the expression of cleaved caspase-3 in RAW264.7 cells compared with that of the control. Moreover, the expression level of cleaved caspase-3 was downregulated in cells pretreated with Z-VAD-FMK (Fig. 4).

It was demonstrated that calcitriol stimulation activated the caspase-3-dependent apoptotic pathway in RAW264.7 cells.

Necrostatin-1 did not suppress the cell death induced by calcitriol

The effect of Necrostatin-1, a necroptosis inhibitor, was examined to determine the calcitriol-induced cell death pathway. The LDH levels in the culture supernatants of

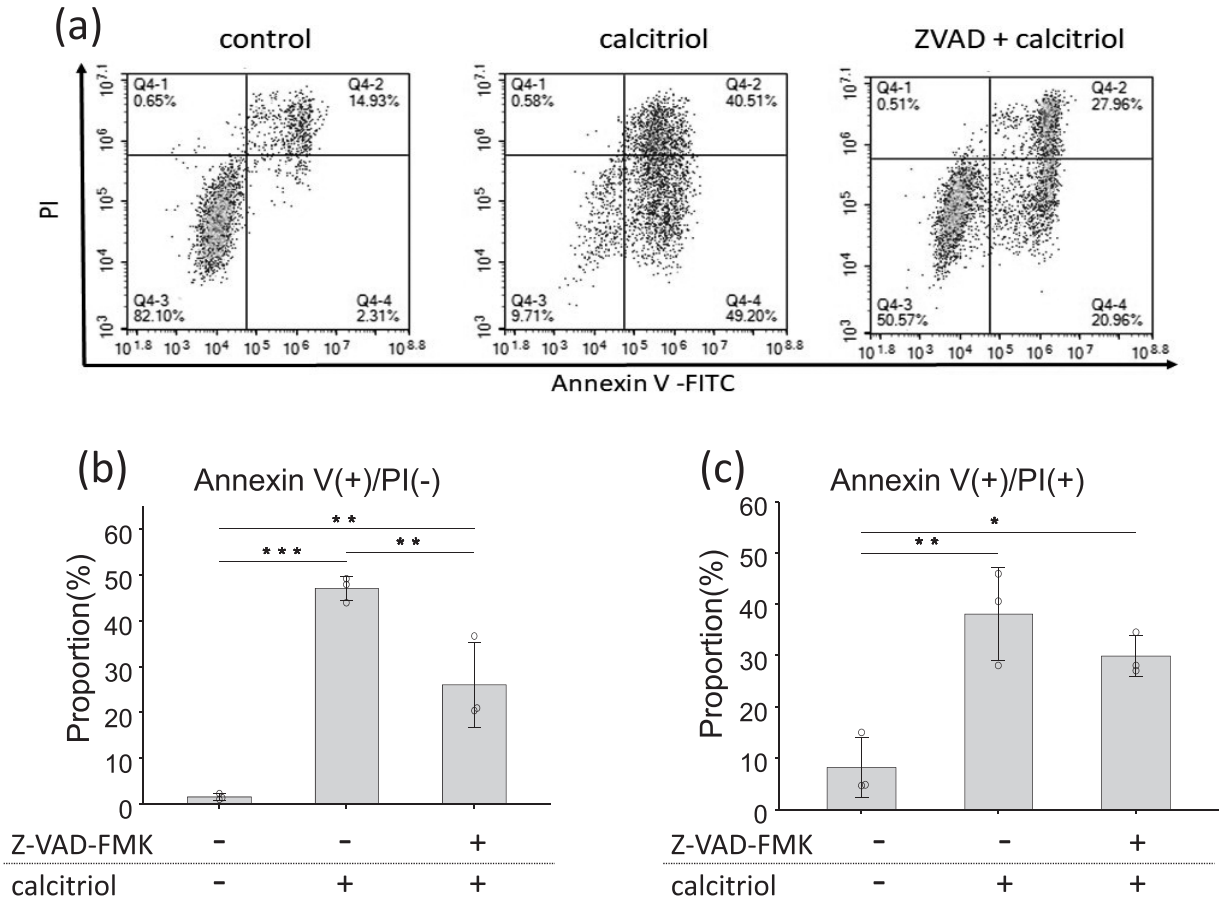

Fig. 3. Flow cytometric analysis of RAW264.7 cells stained with annexin V-FITC/PI. (a) Scatterplots of RAW264.7 cells pretreated with or without Z-VAD-FMK (50 μM) for 2 h, and treated with calcitriol (10 μM) for 3 h. DMSO and ethanol were used as controls. Results are representative of three independent experiments. (b) The percentage of early apoptotic cells (annexin V-positive/PI-negative) was measured using flow cytometry. (c) The percentage of necrotic cells (annexin V-positive/PI-positive) was measured using flow cytometry. Data are presented as mean ± S.D. of the results of three times experiments. Statistical analysis was performed using Tukey's honest significant difference (HSD) test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DMSO, dimethyl sulfoxide; PI, propidium iodide

RAW264.7 cells were significantly increased by stimulation with calcitriol compared with that of the control, and pre-treatment with Necrostatin-1 had no effect on the LDH levels induced by calcitriol (Fig. 5).

Therefore, necroptosis may not be involved in cell death induced by calcitriol stimulation in RAW264.7 cells.

Discussion

In this study, we found that calcitriol stimulation induced cell death in RAW264.7, macrophage-like cells derived from tumors induced by murine leukemia virus, and the mechanism of cell death was related to apoptosis, although several studies have reported that calcitriol protects against cell death in some types of cancer cells[17, 18]. Wang et al. have provided that $1,25(OH)_2D_3$ (100 nM) prevented the apoptosis of HL-60 cells induced by etoposide[17]. Antony et al. also reported that calcitriol (100 nM) had an effect on protecting human acute lymphoblastic leukemia cells (RS4;11 and Sup-B15) from the cell death induced by dexamethasone[18]. This discrepancy might be due to differences in the type of cells and concentration of calcitriol used for stimulation[13–18]. In this study, RAW264.7 cells were used as one of the tumor-derived cells, but it will be necessary to investigate the effects of calcitriol on human cancer cells as well in future study.

Although there are some studies suggesting that calcitriol prevents cell death in leukemia cells as described, this study showed that calcitriol induces cell death in RAW264.7 cells. Calcitriol stimulation induced morphological changes in RAW264.7 cells compared with that of the control, suggesting that calcitriol induced cell

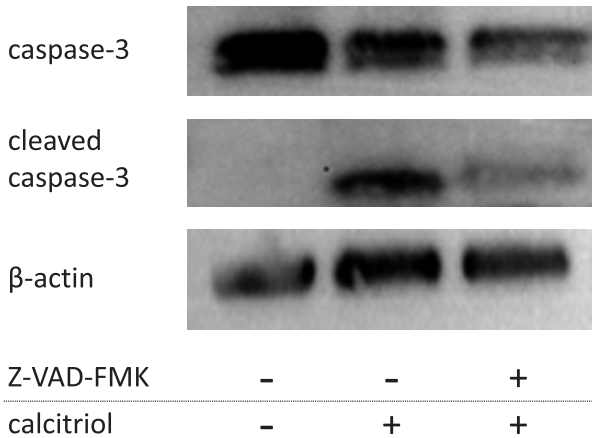

Fig. 4. Expression levels of caspase-3 and cleaved caspase-3 in RAW264.7 cells stimulated with calcitriol. RAW264.7 cells were pretreated with or without Z-VAD-FMK (50 μM) for 2 h and subsequently stimulated with calcitriol (10 μM) for 3 h. Control cells were treated with DMSO for 2 h, then treated with ethanol for 3 h. Proteins in RAW264.7 cell lysates were analyzed using Western blotting. The results are representative of three independent experiments. DMSO, dimethyl sulfoxide

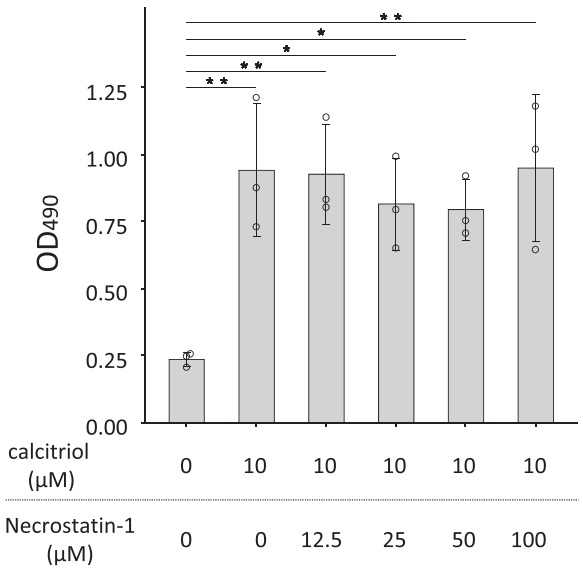

Fig. 5. The effect of Necrostatin-1 on the amount of LDH in the supernatants of RAW264.7 cells stimulated with calcitriol. RAW264.7 cells were pretreated with various concentrations of Necrostatin-1 for 2 h and stimulated with calcitriol (10μM) for 3 h. Necrostatin-1 was diluted with DMSO and the amount of DMSO at each concentration of Necrostatin-1 was equal to that of the control. Control cells were treated with DMSO for 2 h and treated with ethanol for 3 h. The LDH levels were expressed as OD490 (optical density at 490 nm). Data are presented as mean ± S.D. of triplicate assays. Statistical analysis was performed using Tukey's honest significant difference (HSD) test. * $p < 0.05$, ** $p < 0.01$. DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase

death (Fig. 1). It was also shown that calcitriol stimulation increased the LDH levels in the supernatants of RAW264.7, indicating that calcitriol stimulation induced cell death (Fig. 2). The results obtained using flow cytometry showed that calcitriol stimulation increased the percentage of early apoptotic cells to necrotic cells (Fig. 3a), suggesting that apoptosis is involved in calcitriol-induced cell death. Consistent with these results, calcitriol stimulation increased the expression of cleaved caspase-3 in RAW264.7 cells. Cleaved caspase-3 is involved in apoptosis and is the apoptotic executioner[19, 20]. The previous study has demonstrated that metabolic regulation by $1,25(OH)_2D_3$ facilitates increased reactive oxygen species (ROS) and higher oxidative stress in transformed epithelial cells[21]. In addition, Min-Tao et al. have shown that the exposure of cancer cells to $1\alpha, 25(OH)_2D_3$ resulted in elevated levels of ROS, and also induced apoptosis, compared to non-exposed cells[22]. Accumulation of intracellular ROS leads to disruption of the mitochondrial membrane potential, release of cytochrome *c* with subsequent activation of the caspase cascade, and ultimately to programmed cell death through apoptosis[23]. In addition, these pathways are intrinsic pathways of apoptosis and are involved in the Bcl-2 family of mitochondrial proteins[20]. Previous studies have shown that calcitriol-induced apoptosis is caused not only by the upregulation of caspase expression[16] but also by the downregulation of Bcl-2 and upregulation of Bax and Bak expression[12, 15, 24, 25]. Bcl-2 is an anti-apoptotic protein, whereas Bax and Bak are pro-apoptotic proteins and those are Bcl-2 family proteins[12, 20]. Therefore, cell death induced by calcitriol stimulation in RAW264.7 cells might be related to caspases as well as the expression of Bax, Bak, and Bcl-2. Further studies are needed to elucidate whether Bcl-2 family proteins related to apoptosis in RAW264.7 cells are induced by calcitriol stimulation.

Pre-treatment with the pan-caspase inhibitor, Z-VAD-FMK, suppressed the morphological changes, the LDH levels in the culture supernatants, the increase in the percentage of early apoptotic cells, and expression of cleaved caspase-3 caused by calcitriol in RAW264.7 cells (Fig. 1–4). However, calcitriol treatment induced both apoptosis and necrosis (Fig. 3). Moreover, Z-VAD-FMK did not completely inhibit the percentage of early apoptotic to necrotic cells caused by calcitriol stimulation. Therefore, we hypothesized that calcitriol stimulation induces other mechanisms of cell death, including

apoptosis and necrosis.

We investigated whether the necroptosis inhibitor, Necrostatin-1, inhibits cell death induced by calcitriol stimulation, as we considered that calcitriol stimulation induces cell death other than apoptosis and necrosis, as previously described. Necroptosis is a recently discovered form of cell death[26]. Similar to necrosis, it is characterized by the morphology of necrosis, including cell swelling and rupture; and similar to apoptosis, it is controlled by a specific signaling pathway. Therefore, it is also called regulated necrosis. The critical mechanism underlying necroptosis involves the activation of receptor-interacting protein kinase1 (RIPK1), RIPK3, and mixed-lineage kinase domain-like proteins[20, 26]. It has been reported that Necrostatin-1 inhibits necroptosis in Jurkat cells by inhibiting RIPK1[27]. In this study, Necrostatin-1 did not suppress the increase in LDH levels in calcitriol-stimulated supernatants (Fig. 5). Thus, necroptosis may not be involved in the calcitriol-induced cell death pathway in RAW264.7 cells.

On the other hand, it has been reported that when the extrinsic pathway of apoptosis involving death receptors is inhibited by Z-VAD-FMK, apoptosis is inhibited but cell death by necrotic and autophagy is induced. In addition, this necrotic death and autophagy are also related to the production of ROS[28]. Although it is unclear whether the extrinsic pathway of apoptosis is involved, this may be one of the reasons why Z-VAD-FMK could not completely inhibit cell death in this study.

Cell death pathways have long been considered to function in parallel with little or no overlap. However, it is currently clear that apoptosis and necroptosis are closely associated and can interact with each other[20]. Further studies are required to elucidate the cell death pathway in RAW264.7 cells.

There have been some reports on the effects of calcitriol on macrophages. Calcitriol is capable of promoting the differentiation of monocytes into macrophages, and it also reduces the production of inflammatory factors such as IL-1β, IL-6, TNF-α, RANKL, and COX-2 in macrophages. Calcitriol can also induce the production of the anti-inflammatory factor IL-10. Therefore, calcitriol induces anti-inflammatory activity in macrophages[29]. There is another effect of calcitriol in macrophages, that is, calcitriol induces the production of cathelicidin and thus contributes to antibacterial activity[30].

Vitamin D also has a variety of effects on tumor microenvironment (TME) constituent cells, particularly tumor-associated macrophages (TAMs), which are an important component of the TME and account for up to 50 % of tumor cell composition[31]. Generally, M1 macrophages within the TME exhibit anticancer activity. As cancer progresses, M2 macrophages become predominant, promoting tumor growth, enhancing metastatic potential, and contributing to vascular proliferation and remodeling of the tumor stroma[31].

Studies on ovarian cancer have shown that M2 macrophage supernatants induce proliferative and migratory potential of ovarian cancer cells, but calcitriol attenuates these effects. In addition, calcitriol has been reported to reduce the number of M2 macrophages in vitro, and meta-analysis also revealed that a high percentage of M2 macrophages is associated with poor prognosis in ovarian cancer patients[32].

On the other hand, in a study of breast cancer, it has been reported that the administration of calcitriol to mice fed a vitamin D-deficient diet induces the polarization of TAMs to M2 macrophages, and as a result, promotes the lung metastasis of 4T1 breast cancer cells[31]. However, it was also stated that calcitriol or vitamin D has various effects on the microenvironment of macrophages and tumors, and that these effects differ depending on the group of patients examined[31]. Just as the effects of calcitriol on cancer cells vary, the effects of calcitriol on TAMs also vary, and are thought to depend on the type of cancer cell and the surrounding environment. In this research on breast cancer, the mice were treated with calcitriol (1 μg/kg), and then the experiments were conducted using breast cancer cells and TAMs derived from these mice. As a result, it was found that calcitriol had a tumor-promoting effect. Taken together, although there are many effects of calcitriol on cancer, we consider that it may be possible to lead tumor-associated cells to apoptosis if it is possible to treat them directly with calcitriol in high concentrations (10 μM), as treated in this research.

Epidemiological studies have shown that vitamin D levels may be associated with the development and progression of cancer. There are many epidemiological studies that demonstrate the relationship among cancer, vitamin D, and sunlight[4, 33–39]. In 2006, Giovannucci et al. developed a method for predicting the 25-hydroxy-vitamin D (25(OH)D) levels from the geographic region, skin pigmentation, dietary intake, supplement intake, body mass index, and leisure-time physical activity[40]. They showed that the predicted 25(OH)D level was associated

with a 17% reduction in total cancer incidence, a 29 % reduction in total cancer mortality, and a 45 % reduction in digestive system cancer mortality[40]. Many studies have demonstrated that plasma 25(OH)D levels are associated with the incidence and mortality rates of cancer including melanoma, lung cancer, breast cancer, prostate cancer, colon cancer, ovarian cancer, kidney cancer, esophageal cancer, stomach cancer, and non-Hodgkin's lymphoma[4, 33, 37, 41, 42]. These studies strongly support the hypothesis that high plasma 25(OH)D levels decrease cancer risk. Therefore, the prospective effect of vitamin D against cancer has been established. However, calcitriol has not been used as an anti-cancer drug because of its side effects, such as hypercalcemia. Although there is a risk of hypercalcemia, recently, vitamin D and its analogs have been administered for cancer treatment in clinical trials[43–46]. Additionally, novel vitamin D analogs that can avoid hypercalcemia have been developed[8, 24, 47, 48]. Therefore, vitamin D may be used as an anti-cancer drug in the future. To the best of our knowledge, we found for the first time that calcitriol induces apoptosis in RAW264.7 cells. Elucidating the pathway of cell death induced by calcitriol in RAW264.7 is a promising tool for the future use of vitamin D in cancer treatment.

Conclusion

Calcitriol stimulation induced changes in the shape of cells, increasing the LDH levels in the culture supernatants, increasing the percentage of cells stained with both annexin V and PI and the expression of cleaved caspase-3 in RAW 264.7 cells. In addition, Pre-treatment with Z-VAD-FMK suppressed these effects of calcitriol. Moreover, pre-treatment with Necrostatin-1 did not decrease the LDH levels in the supernatants of RAW264.7 cells stimulated with calcitriol. Therefore, the present study has clarified that calcitriol stimulation induced apoptosis and did not induce necroptosis in RAW264.7 cells. Thus, vitamin D might be a good candidate for anti-cancer drugs.

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Conflict of interest

The authors have asserted no conflict of interest relevant to this article.

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