의학박사 학위 논문

난소암 세포주에서 Calcitriol 과 백금 항암제의 병합 효과

Effects of calcitriol combined with platinum-based chemotherapy in ovarian cancer SKOV-3 cells

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의 학 과

김 주 현

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지 도 교 수　김 용 만

이 논문을 의학박사 학위 논문으로 제출함

2020년 12월

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김주현의 의학박사 학위 논문을 인준함

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2020년 12월

Abstract

Effects of calcitriol combined with platinum-based chemotherapy in ovarian cancer SKOV-3 cells

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Objective: To evaluate the effects of combination calcitriol and chemotherapy; to identify molecular mechanisms whereby calcitriol affects ovarian cancer cells.

Materials & Methods: I treated SKOV-3 cells lines with calcitriol and cisplatin in a dose-dependent manner and compared their effects alone and in combination. I assessed cell viability, cell proliferation, and apoptosis using the following assays: PrestoBlue, intracellular ATP, caspase-3/7 activity, annexin V, and immunoblotting.

Results: Calcitriol alone caused dose-dependent inhibition of cell survival and proliferation. Calcitriol induced apoptotic cell death by increasing caspase-3/7 activity and activating apoptosis-related molecules such as cleaved caspase-3 or cleaved poly (ADP-ribose) polymerase (PARP). However, the inhibition began at 10 μmol L^{-1}, a concentration higher than the physiologically known concentration of calcitriol. Combination treatment using calcitriol at a physiological concentration of 10-100 nmol L^{-1} plus cisplatin significantly suppressed cell survival. Further, significantly increased apoptotic cell death and caspase-3 activity, downregulated the expression of B-cell lymphoma 2 (Bcl-2) and vascular endothelial growth factor, and upregulated the expression of vitamin D receptor, Bcl-2-associated X-protein (Bax), cleaved caspase-3, and cleaved PARP.

Conclusion: In ovarian cancer SKOV-3 cells, calcitriol plus cisplatin had greater antiproliferative, apoptotic, and anti-angiogenic effects than cisplatin

alone. Adding calcitriol to platinum-based chemotherapy might be beneficial to patients with ovarian cancer.

Key word: Vitamin D, calcitriol, in vitro test, platinum-based chemotherapy, growth inhibition, VDR, vascular endothelial growth factor

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Introduction

Ovarian cancer is the fifth leading cause of cancer death and is the leading cause of mortality in women.[1] The five-year survival rate for women with epithelial ovarian cancer (EOC) is less than 40% because, in most cases, the disease has already advanced at the time of diagnosis.[2,3] Patients with advanced EOC undergo cytoreductive surgery and platinum-based chemotherapy; those who receive adjuvant chemotherapy or preoperative chemotherapy will also undergo cytoreductive surgery.[2,4] Patients with advanced EOC respond well to initial platinum-based therapy, but the disease eventually recurs in more than 75% of patients.[5] Patients with recurrent EOC receive additional chemotherapy, which increases the survival rate but does not ultimately cure the disease. The treatment for ovarian cancer, which is based on cytoreductive therapy combined with platinum-based chemotherapy, has not significantly progressed over the past few decades, so there is growing interest in new approaches for EOC treatment.[6] The growing biological understanding of EOC has led to the development of several target molecules and biological treatments, including angiogenesis inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, and immunotherapy.[7]

Vitamin D is a fat-soluble prohormone with a well-established role in maintaining calcium and bone homeostasis. Nearly 3% of the human genome is regulated by the vitamin D endocrine system.[8] Calcitriol is an activated hormone of vitamin D that binds to the vitamin D receptor (VDR) in the cell nucleus.[9,10] Studies have described several mechanisms through which VDR can inhibit tumor growth, including genomic and non-genomic signal transduction.[8,10,11] In addition, calcitriol can regulate important biological processes such as insulin secretion, immune responses, cell differentiation, antiproliferation, apoptosis, angiogenesis inhibition, invasion and metastasis inhibition, and anti-inflammatory actions.[9,12-15]

Many VDR genes have been identified in ovarian cancer cells.[16] Further,

calcitriol has been reported to induce cell cycle arrest and apoptosis in ovarian cancer cells.[17-19] However, the main mechanisms involved in calcitriol activity in ovarian cancer have not been revealed, and the effect of its combination with anticancer drugs has not been studied. Therefore, I aimed to investigate the potential effects and mechanisms of action, in ovarian cancer cells vitro, of calcitriol alone and in combination with cisplatin, a platinum-based anticancer drug that is the core ovarian cancer treatment.

Materials and Methods

1. Cell culture and drug

The human ovarian cancer cell line SKOV-3 was purchased from the American Type Culture Collection (Rockville, MD, USA). The cell line was cultured in McCoy's 5A medium (Welgene, Kyungsan, Republic of Korea) containing 10% fetal bovine serum (FBS) and 1% penicillin - streptomycin (P/S; Thermo Fisher, CA, USA), in a humidified chamber with 5% CO2 at 37 °C. Calcitriol and cisplatin were purchased from Selleckchem (Houston, USA), and the reagents were dissolved in dimethyl sulfoxide. The final concentrations in the culture medium did not exceed 0.1%.

2. Cell viability

Cell viability was assessed using PrestoBlue cell viability reagent (Thermo Fisher, CA, USA). SKOV-3 cells were plated in 96-well plates in McCoy's 5A completed medium (containing 10% FBS and 1% P/S) and treated with calcitriol or cisplatin at concentrations of 0 - 100 μmol L^{-1} for 48 h. The treated cells were mixed with 10% PrestoBlue reagent in culture medium, and incubated for 60 min at room temperature. All data are expressed as percentages, relative to the control.

3. Cell proliferation

Cell proliferation was measured by evaluating ATP content, using the CellTiter-Glo assay kit (Promega, WI, USA). The cells were seeded in McCoy's 5A completed medium and treated with calcitriol or cisplatin (0 - 100 μmol L^{-1}) for 48 h. Equal volumes of CellTiter-Glo reagent and cell culture medium were placed in each well. The cells were incubated at room temperature for 10 min, and the luminescence signal was recorded using a luminescence plate reader (Berthold, Bad Wildbad, Germany).

4. Caspase 3/7 activity

The cells were cultured for 24 h in a white-walled 96-well plate and treated with calcitriol or cisplatin (0 - 100 μmol L^{-1}) for 48 h. The treated cells were incubated with 100 μL of Caspase-Glo 3/7 reagent at room temperature for 30 min. Luminescence was measured using a luminometer (Berthold, Bad Wildbad, Germany). Results are expressed as fold induction, relative to the control.

5. Annexin V-FITC/PI assay by flow cytometry

The apoptotic cell death rate was determined using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, CA, US) according to the protocols provided. The cells were seeded in 6-well plates, and were treated with calcitriol or cisplatin (0 - 100 μmol L^{-1}) for 48 h. The supernatant and cells were harvested and centrifuged at 1500 rpm for 7 min. The cell pellet was resuspended using an FITC Annexin V and PI mixture (comprising 1× binding buffer, FITC Annexin V, and PI), incubated for 15 min at room temperature (25 °C) in the dark. After incubation, 1× binding buffer was added to each sample, and the samples were analyzed by flow cytometry within 1 h. Flow cytometric analysis was carried out using a FACS Calibur flow cytometer (BD Biosciences, CA, USA). Results are presented as percentages of the total number of gated cells.

6. Short interfering RNAs and transfection

short interfering (si) RNAs for human vitamin D receptor was synthesized from Genolution (Genolution Pharmaceutical Inc, Seoul, Republic of Korea), and siRNA for control (SCR, sc-37007) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The VDR-targeting sequence, sense: 5'-GGAGUUCAUUCUGACAGAU-3'. The transience transfection experiment with silencing and scrambled VDR was performed using Lipofectamine RNAi

MAXTM according to the manufacturer's instruction (Thermo Fisher, CA, USA). And the transfected cells were cultured in humidified chamber with 5% CO2 at 37°C for 48~72 hours until performing another assays.

7. Vascular endothelial growth factor (VEGF) activity

The cells were incubated with or without calcitriol for 48 h at 37°C. VEGF activity was evaluated using a VEGF Human ELISA Kit (Thermo Fisher Scientific, CA, USA), according to the manufacturer's protocol. Absorbance was measured at 450 nm (Bio Rad Laboratories, CA, USA).

8. Western blot

The cells were harvested and resuspended in ice-cold cell lysis buffer (Intron Biotechnology, Seongnam, Republic of Korea) containing a Complete Protease Inhibitor Cocktail Mini tablet (Roche Diagnostics, Seoul, Rebuplic of Korea). Protein concentrations were determined using a Bicinchoninic acid assay kit (Pierce, IL, USA) according to the manufacturer's instructions. Proteins were separated using 12% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and blocked with 5% skim milk. Membranes were incubated with anti-VDR, anti-B-cell lymphoma 2 (Bcl-2), anti-VEGF (Abcam, CB, UK), anti-Bcl-2-associated X (Bax), anti-cleaved caspase-3, anti-PARP, anti-phosphatidylinositol 3 kinase (PI3K), anti-Protein kinase B (AKT), anti-phospho-AKT (Ser 473), anti-phospho AKT (Thr 308), anti-mammalian target of rapamycin (mTOR), and beta-actin (Cell Signaling Technology, MA, USA) antibodies in 5% skim milk or 1% bovine serum albumin. Next, the membranes were incubated with Horseradish Peroxidase-conjugated anti-secondary IgG antibody (Cell Signaling Technology, MA, USA) and visualized using SuperSignal West Pico Chemiluminescent Substrate (millipore, MA, USA).

9. Statistical analysis

All data are presented as mean ± SD. Student's t-tests were used to determine the significance of differences between different groups. $P < 0.05$ was considered statistically significant.

Results

Calcitriol suppresses cell survival and induces apoptotic cell death in ovarian cancer cell

To study the effects of calcitriol on the survival of human ovarian cancer cell lines, we first performed cell viability and proliferation analysis. The cells were treated with calcitriol at different concentrations (0 to 100 μmol L^{-1}) for 48 h, and then cell viability or proliferation were assessed using PrestoBlue or Cell Titer-Glo assays. As shown in Figure 1A and 1B, calcitriol dramatically suppressed the cell viability (Figure 1A) and proliferation (Figure 1B) of SKOV-3 cells in a dose-dependent manner. To further confirm the induction of apoptosis by calcitriol in human ovarian cancer cells, we performed analysis of apoptosis activity and apoptosis signaling pathway. The cells treated with calcitriol assessed using Caspase-3/7 activity assays, and calcitriol significantly increased caspase-3 activity in a dose-dependent manner (Figure 1C). Also, calcitriol increased expression of pro-apoptotic protein cleaved caspase-3 and cleaved PARP and suppressed expression of anti-apoptotic protein Bcl-2 (Figure 1D). These results demonstrated that calcitriol might inhibit survival of cancer and induce apoptotic cell death in ovarian cancer cells.

Combination therapy calcitriol with cisplatin represses cancer survival and promotes apoptotic cell death in an ovarian cancer cell

To test the hypothesis that addition of calcitriol to therapy using platinum-based anticancer drug would reduce cancer survival and induce apoptotic cell death in ovarian cancer cell, the SKOV-3 cells were co-treated with 0-50 μmol L^{-1} calcitriol and 10 μmol L^{-1} cisplatin for 48h. The cell treated with combination therapy exhibited the largest decrease of cell viability (Figure 2A) and proliferation (Figure 2B) in calcitriol 10 nmol L^{-1}/cisplatin 10 μmol L^{-1}or calcitriol 100nM/cisplatin 10μM compared with

single-agent calcitriol or cisplatin. Moreover, when we treated two doses of calcitriol/cisplatin (10 nmol L^{-1}/10 μmol L^{-1} or 100 nmol L^{-1}/10 μmol L^{-1}) for 48h in the cells, the caspase-3 activity (Figure 2C) and the apoptotic cells (Figure 2D) were decreased in combined calcitriol/cisplatin compared with calcitriol or cisplatin monotherapy in SKOV-3 cells. Western blot analysis showed that the combination therapy promoted expression of pro- or anti-apoptotic molecule genes than calcitriol or cisplatin monotherapy with dose-dependent manner in ovarian cancer cells (Figure 2E). Collectively, these data revealed that combination therapy of calcitriol and cisplatin induced apoptotic cell death in human ovarian cancer cell.

Inhibition of cell survival through regulation of vitamin D activity

To determine whether the activity of vitamin D contributes to regulate the survival cancer cell, we treated the calcitriol, cisplatin or calcitriol/cisplatin in siVDR-transfected ovarian cancer cells. The knockdown of VDR inhibited calcitriol/cisplatin-induced cell viability (Figure 3A) and proliferation (Figure 3B) in SKOV-3 cell. But VDR Knockdown was not affected the cisplatin-induced cell viability and proliferation in SKOV-3 cell. Also, when we analyzed apoptotic cell death (Figure 3D) and measured the caspase-3 activity (Figure 3C) using luciferase assay or flow cytometry, the knockdown of VDR was significantly suppressed calcitriol/cisplatin-induced apoptotic cell death and caspase-3 activity in SKOV-3 cell. Thus, our research findings indicate that the regulation of vitamin D activity by VDR knockdown mediated calcitriol/cisplatin-stimulated cell survival in ovarian cancer cells.

Suppression of VEGF activity by vitamin D

To determine whether vitamin D activity contributes to regulate angiogenesis mechanism, we performed the ELISA assay in calcitriol-, cisplatin- or calcitriol/cisplatin-treated ovarian cancer cells. As shown in Figure 4, the calcitriol decreased the VEGF activity in SKOV-3 cell (Figure 4A, 4B).

Besides, the combination therapy of calcitriol and cisplatin promoted the reduction of VEGF activity in SKOV-3 cell (Figure 4C, 4D). Thus, our results demonstrated that vitamin D mediated angiogenesis signaling pathway through regulation of VEGF activity in ovarian cancer.

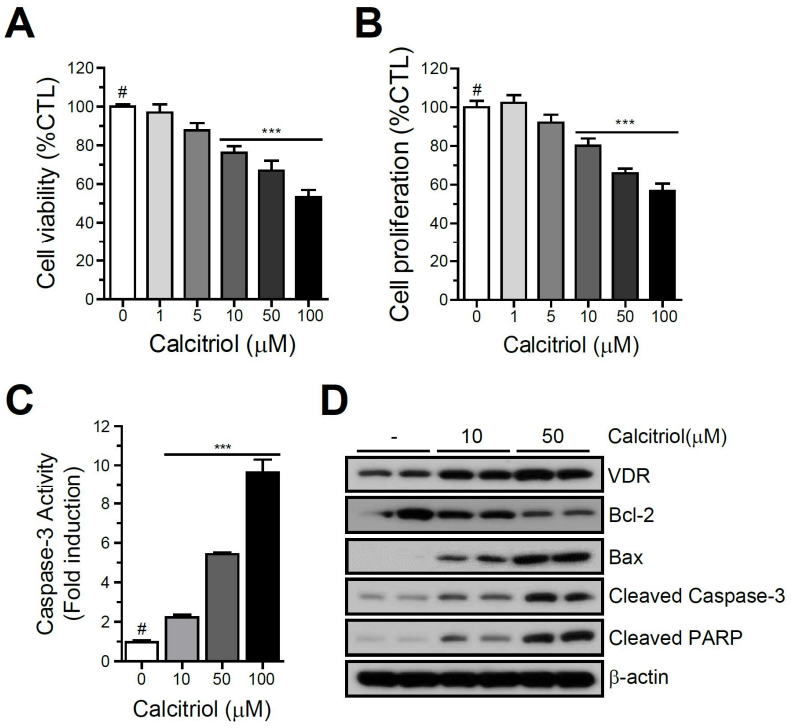

Figure 1. Inhibition of cell survival and induction of apoptotic cell death by calcitriol in SKOV-3 cells. (A) SKOV-3 cells were cultured with calcitriol at 0 - 100 μmol L^{-1} for 48h, and cell viability was detected by PrestoBlue assay. (B) The proliferation of calcitriol-treated SKOV-3 cells was determined by measuring adenosine triphosphate content, using the CellTiter-Glo assay. (C) Caspase-3 activity in calcitriol-treated SKOV-3 cells was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. (D) Western blot analysis showing the presence of vitamin D receptor (VDR), Bcl (B-cell lymphoma) 2, Bax (Bcl-2-associated X), cleaved caspase 3, and cleaved poly (ADP-ribose) polymerase (PARP), in calcitriol-treated cells. Data are expressed as means ± standard deviation. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with the controls. The controls were treated with 0.1% dimethyl sulfoxide. %CTL: percentage relative to the level in the control.

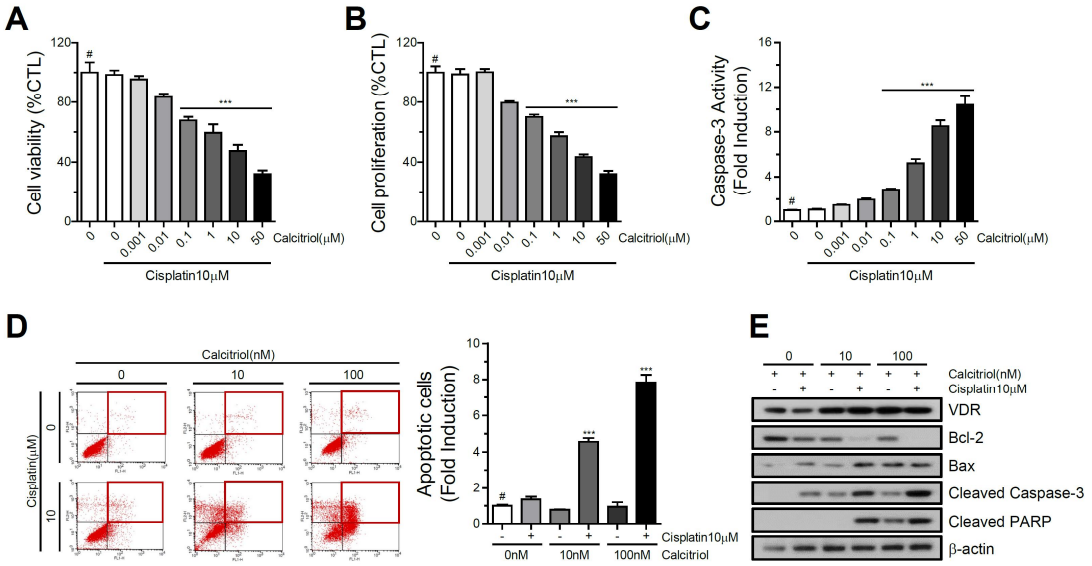

Figure 2. Effect of combination calcitriol and cisplatin therapy on cell survival and apoptotic cell death in SKOV-3 cells. SKOV-3 cells were cotreated with combination 10 μmol L^{-1}cisplatin and 0 - 50 μmol L^{-1}calcitriol for48h. Cell viability (A) and proliferation (B) were measured using PrestoBlue or CellTiter-Glo assays, respectively. (C) Caspase-3 activity in combination cisplatin and calcitriol-treated SKOV-3 cells was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. (D) Flow cytometric analysis of Annexin V FITC/Propidium Iodide stained SKOV-3 cells treated with combination 10 μmol L^{-1}cisplatin and 0,10, and 100 nmol L^{-1}calcitriol for 48h. Quantitative analysis of the apoptosis rates of SKOV-3 cells under various concentrations of calcitriol. (E) Western blot analysis showing the presence of vitamin D receptor (VDR), Bcl (B-cell lymphoma) 2, Bax (Bcl-2-associated X), cleaved caspase 3, and cleaved poly (ADP-ribose) polymerase (PARP), in calcitriol-treated cells. Data are expressed as means ± standard deviation. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with the controls. The controls were treated with 0.1% dimethyl sulfoxide. %CTL: percentage relative to the level in the control.

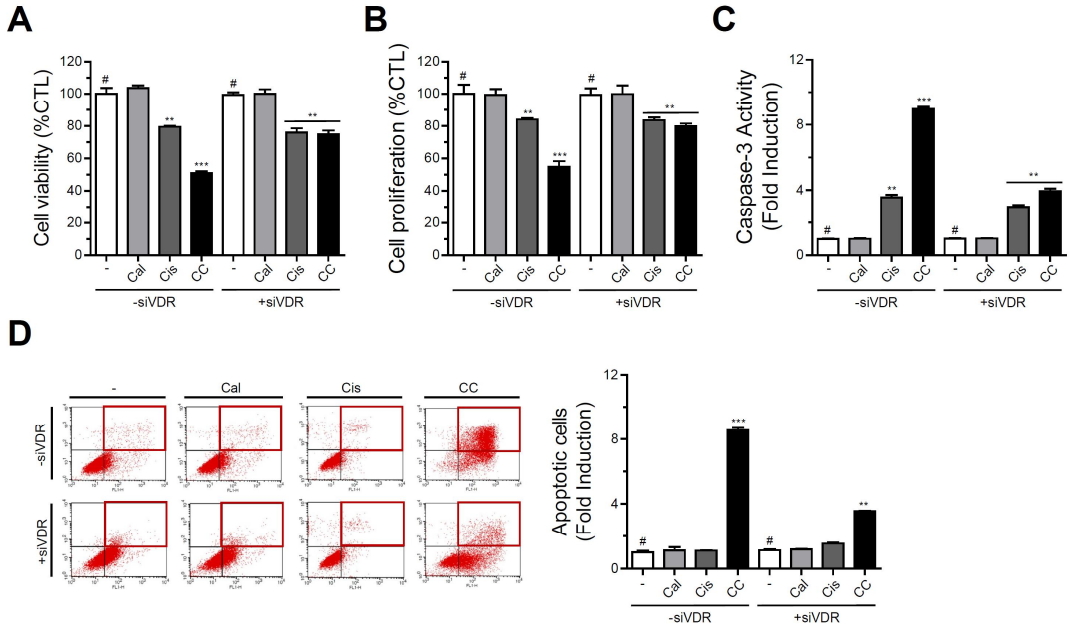

Figure 3. Effect of combination calcitriol and cisplatin therapy on cell survival and apoptotic cell death via regulation of vitamin D receptor (VDR) expression in SKOV-3 cells. SKOV-3 cells were transfected with scrambled (SCR) or silencing (si) VDR, and treated with 100 nmol L^{-1} calcitriol, 10 μ mol L^{-1} cisplatin, or combination 100 nmol L^{-1} calcitriol and 10 μmol L^{-1} cisplatin, for 48h. (A) Cell viability and (B) cell proliferation were measured using PrestoBlue or CellTiter-Glo assays, respectively. (C) Caspase-3 activity, in single-agent or combination therapy SCR- or VDR-knockdown SKOV-3 cells, was measured by luciferase assay, using the Caspase-Glo 3/7 Assay system. (D) Flow cytometric analysis of Annexin V FITC/Propidium Iodide stained single-agent or combination therapy SCR- or VDR-knockdown SKOV-3 cells. Data are expressed as means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with the controls. Controls were treated with 0.1% DMSO. %CTL: percentage relative to the level in the control.

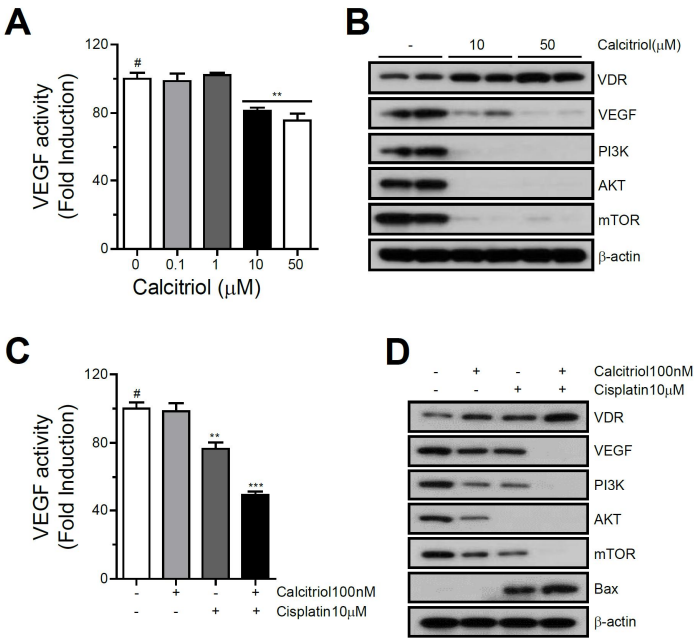

Figure 4. Inhibition of vascular endothelial growth factor (VEGF) activity by combination calcitriol and cisplatin therapy in SKOV-3 cells. (A) VEGF activity was determined via ELISA assay in SKOV-3 cells treated with calcitriol alone. (B) Western blot analysis showing the presence of vitamin D receptor (VDR), vascular endothelial growth factor (VEGF), phosphatidylinositol 3 kinase (PI3K), Protein kinase B (AKT), and mammalian target of rapamycin (mTOR) in SKOV-3 cells treated with calcitriol alone. (C) VEGF activity was determined using ELISA assay in SKOV-3 cells treated with combination calcitriol and cisplatin. (D) Western blot analysis showing the presence of VDR, VEGF, PI3K, AKT, mTOR, and Bcl-2-associated X (Bax) in cells treated with combination calcitriol and cisplatin $*P < 0.01$, compared with the control group.

Discussion

In this study, the effect of combination calcitriol and cisplatin therapy on the growth of ovarian cancer cells in vitro was verified for the first time. The combination therapy inhibited cancer cell growth more than separate use. Further, calcitriol inhibited cell growth and induced apoptotic cell death via VDR, and inhibited angiogenesis by inhibiting VEGF.

Several epidemiological studies have reported relationships between ovarian cancer and vitamins. The hypothesis that vitamin D is associated with cancer incidence was first proposed in 1980 by the Garland brothers, who reported that people with vitamin D deficiency at higher latitudes have a higher risk of developing malignant tumors.[20] Subsequently, their epidemiological studies confirmed that there is a strong inverse correlation between the average daily amount of UV radiation and the ovarian cancer mortality rate.[21] In addition, in a case-controlled study of 1631 female patients diagnosed with EOC, a higher concentration of 25(OH)D improved higher survival rate.[22] A meta-analysis of 10 cohort studies on the prevalence of ovarian cancer confirmed that an average increase of 20 ng mL^{-1} of $25(OH)_2D_3$ was associated with a relative risk of 0.83 (0.63 - 1.08).[23]

Many experimental studies have reported that calcitriol inhibits the growth of ovarian cancer cells,[18,24,25] and that it increases apoptosis.[17] In the current study, calcitriol alone increased cell growth inhibition and apoptosis (Figure 1). However, at a physiological concentration, its effect was stronger in combination with cisplatin (Figure 2). Moreover, VDR expression increased with calcitriol concentration (Figures 1 and 2). This is consistent with other studies; for instance, high levels of 1,25-dihydroxyvitamin D_3 can increase VDR expression in ovarian cancer cell lines,[17] and other studies have reported that ovarian cancer tissue expresses VDR.[24,26] Therefore, there may be functional vitamin D pathways for the treatment or prevention of ovarian cancer.[27] Further, VDR knockdown reduced the effect of calcitriol (Figure 3),

confirming that calcitriol functions via VDR. This is consistent with Jiang et al.,[18] who reported that calcitriol-induced suppression of cancer growth and increased apoptosis was mediated by VDR.

Further, VEGF activity was inhibited by calcitriol; this inhibition was increased in combination with cisplatin (Figure 4). Vitamin D has been reported to have a potential role in inhibiting tumor angiogenesis.[28,29] An in vivo study reported that vitamin D reduced the inhibition of tumor-derived epithelial cell growth in VDR-knockout mice, and that the loss of VDR increased the levels of hypoxia-inducible factor (HIF)-1α, VEGF, angiopoietin 1, and platelet-derived growth factors.[28] Increased angiogenesis appears to be a process of cell adaptation to hypoxic conditions, which is regulated by HIF1. HIF1 target genes such as VEGF are mediated via HIF-dependent pathways that are inhibited by $1,25(OH)_2D_3$.[29] In clinics, the treatment of advanced and recurrent ovarian cancer involves the use of bevacizumab, a VEFG inhibitor, in combination with platinum. Therefore, the anti-angiogenesis effect of calcitriol may be helpful in the treatment of ovarian cancer.

Conclusion

This study suggests a new treatment approach using vitamin D, which is safe and widely consumed on a daily basis, to reduce recurrence and increase survival in the treatment of ovarian cancer. It may be possible to obtain synergistic effects by adding vitamin D to existing cytotoxic chemotherapy. Moreover, VDR expression may be a good predictive biomarker in patients with ovarian cancer. This study will serve as a cornerstone for future clinical studies.

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국문 요약

목적: 칼시트리올과 백금 항암제를 병용하여 처리 시 인간 난소암 세포주 (SKOV-3)의 체외 성장에 미치는 효과에 대해 조사하고 그 기전을 밝히고자 하였다.

대상 및 방법: SKOV-3 세포주에서 칼시트리올과 시스플라틴을 용량 의존 방식으로 처리하고 각 약물 단독 및 조합의 효과를 비교하였다. Prestoblue assay, 세포 내 ATP 분석, caspase-3/7 활성, annexin V 분석 및 immunoblotting을 사용하여 세포 생존, 세포 증식 및 세포 사멸 정도를 확인하였다.

결과: 칼시트리올 단독으로 처리한 SKOV-3 세포주에서 용량 의존적으로 세포 생존과 증식이 억제되었고 세포 사멸이 증가하였다. 그리고 칼시트리올 용량 의존적으로 비타민 D 수용체의 발현이 증가함을 확인하였다. 칼시트리올과 시스플라틴의 병합 처리 결과 칼시트리올의 생리학적인 농도에서 세포 생존과 증식이 현저하게 억제가 되었으며, 세포 사멸이 증가하였다. 그리고 B-cell lymphoma 2 (Bcl-2)의 발현이 하향 조절되었고, cleaved caspase-3 및 cleaved poly (ADP-ribose) polymerase (PARP) 그리고 Bcl-2-associated X-protein (Bax)의 발현은 상향 조절되었다. 그리고 칼시트리올 단독 처리 시 vascular endothelial growth factor (VEGF) 활성도가 용량 의존적으로 감소하였고, 시스플라틴과 병합 시 생리학적인 농도에서 현저하게 VEGF 활성도가 감소하였다.

결론: 난소암 세포주 (SKOV-3)에서 시스플라틴과 병합된 칼시트리올은 시스플라틴 단독보다 우수한 세포사멸, 항증식 및 항혈관 신생 효과를 나타내었다.

중심 단어: 비타민 D, 칼시트리올, 체외 실험, 백금항암제, 성장억제, 비타민 D 수용체, 혈관 내피 성장 인자