농학 석사 학위논문

Curcumin induced-apoptosis through inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity in ovarian cancer cells

난소암 세포주에서 curcumin에 의해 유도되는 sarco/endoplasmic reticulum Ca^{2+} ATPase 활성억제를 통한 세포사멸 기전 연구

2014 년 7 월

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이 논문을 농학 석사학위논문으로 제출함

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서 정 아

서정아의 석사학위논문을 인준함

2014년 8월

위 원 장 (인)

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Curcumin induced-apoptosis through inhibition of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity in ovarian cancer cells

By

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Approved by Thesis Committee:

Professor Jeong M. Lim Chairman

Professor _____ Vice chairman

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ABSTRACT

Sarco/endoplasmic Reticulum Calcium ATPase (SERCA) is a calcium ATPase pump which is on the endoplasmic reticulum (ER) membrane, regulating the Ca^{2+} homeostasis. In addition, it is reported that SERCA2 mRNA is elevated in ovrarian cancer. In this study, we demonstrated that curcumin increased the concentration of cytosolic Ca^{2+} through inhibition of SERCA activity, causing apoptosis only in ovarian cancer cells, not in normal cells (PBMC, peripheral blood monocyte: OSE, ovarian surface epitheral cell). Curcumin induced-apoptosis in ovarian cancer cells was concentration- and time- dependent. Cytosolic Ca^{2+} flux was immediately increased after the curcumin treatment and persisting at the high level till 48 h. Treatment of BAPTA-AM, a Ca^{2+} chelator, reduced curcumin induced-apoptosis, which confirmed the importance of intra-cytosolic Ca^{2+} concentration. There was no difference basal mRNA and protein expression of SERCA 1 and SERCA3 between normal ovarian epithelial cell and ovarian cancer cells. Protein level of SERCA2 was significantly higher in cancer cells than in normal cells, while mRNA level of SERCA2a and b were not different between normal and cancer cells. The SERCA activity was suppressed by curcumin without any change of the expression level of protein. The transfection of SERCA2b gene into ovarian cancer cells prevented curcumin induced cytosolic Ca^{2+} elevation with subsequent apoptosis, verifying the important role of SERCA for survival of ovarian cancer cells. Taken together,

inhibition of SERCA activity by curcumin disrupts the Ca^{2+} homeostasis and promotes apoptosis in ovarian cancer cells.

Keywords: curcumin, apoptosis, calcium, SERCA, ovarian cancer
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LIST OF ABBREVIATIONS

SERCA : Sarco/endoplasmic Reticulum Calcium ATPase

ER : endoplasmic reticulum

PBMC : peripheral blood monocyte

OSE : ovarian surface epitheral cell

BAPTA-AM : 1,2-Bis (2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PARP : Poly ADP ribose polymerase

INTRODCUTION

Calcium is one of the major signaling molecules to regulate numerous physiological processes including cell proliferation[1] and apoptosis[2]. Elevated cytosolic Ca^{2+} gives the cell benefit of avoiding apoptosis and of cell survival, therefore, various cancers shows the alterantion of Ca^{2+}-relating signaling[3]. One of the altered Ca^{2+}-relating signaling is sarco/endoplasmic reticulum calcium ATPase (SERCA). SERCA is one of the major Ca^{2+} pumps and it regulates cellular Ca^{2+} flux to transfer Ca^{2+} from cytosol into ER[4]. Overexpressed SERCA affects the cellular Ca^{2+} peak and oscillations, and subsequently promotes activation of signaling pathways related with cell survival[5] even in cancer cell survival[6,4] including liposarcoma[7] and colorectal cancer[8]. Therefore, adjust the overexpressed SERCA is one of the efficacious therapeutic approach.

Curcumin is a yellow pigment which is from *Curcuma longa* and well known about its strong anti-cancer activity. It has been published about its strong anti-cancer effects in various tumors[9, 10,11,12] including ovarian cancer[13]. The cell cycle arrest[14] and caspase cascade-mediated apoptosis[15] are already known for the anticancer acitivity of curcumin in ovarian cancer. However, the detailed mechanism of curcumin induced-apoptosis is not fully understood. Inhibition of SERCA activity by curcumin in rabbit skeletal mescle[16] and Cos7 cell[17] is suggested the possibility of curcumin as an effective SERCA inhibitor in cancer cell.

Ovarian cancer is the fifth leading cause of gynecologic cancer related death among the women in worldwide[18,19]. Diagnosed in late stage[20] and high recurrence rate as 80%[21] result in high mortality of ovarian cancer. The recurred ovarian cancer has shown that strongly resistant to the conventional chemo-therapy[22,23]. For these reasons, the 5-year survival rate of ovarian cancer patients is reported only 27%[24] and this rate has not improved in decade.[25] Therefore, it is a pressing need to find efficacious therapy for ovarian cancer. According the study of gene alteration in ovarian cancer, the mRNA level of SERCA is overexpressed[26]. Thus, control the SERCA is one of the efficacious strategy for treatment of ovarian cancer.

Based on the previous studies, we demonstrated that curcumin induces disruption of calcium homeostasis through inhibition of SERCA activity and promotes apoptosis in ovarian cancer cells.

MATERIALS AND METHODS

1. Compounds, Antibodies and material

RPMI 1640, and MEM were obtained from Welgene (Daegu, South Korea). Fetal bovine serum and penicillin-streptomycin were purchased from Gibco ® Life Technologies. (Gaithersburg, MD) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco (USA, Solon, OH), Annexin V-APC/propidium Iodide (PI) apoptosis detection kit was from BD Biosciences (San Jose, CA). Curcumin, thapsigargin, and A23187, ponceau S were obtained from Sigma-aldrich. (St. Louis, MO) BAPTA-AM was from Invitrogen® Life Technologies.(Gaithersburg, MD) SERCA2 (1:2000) and SERCA3 (1:1000) antibodies were from Abcam plc (Cambridge, United Kingdom), PAPR (1:2000) and cleaved caspase 3 (1:2000) and α-tubulin (1:10000) antibodies were from Santa-cruz Biotechnology. (Santa Cruz, CA)

2. Cell culture

MDAH 2774 (p53 mutant type cell) and SKOV3 (p53 null type cell), were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. PA1 (p53 wild type cell) was cultured in MEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2 (Steri-cycle CO_2 incubotor, Thermo scientific, Waltham, MA).

3. Cytotoxicity Assay

Cytotoxicity of curcumin was determined by MTT assay. Cells were grown on 96 well-plate culture dish for 24 h. The cell-grown medium was discarded and 100 ㎕ of media which was treated to a range of different concentrations of curcumin for 24 h and 48 h. Fifty micro-liter of 0.4% MTT solution was added on the curcumin containing media and incubated at 37 ℃ for 3 h. MTT was removed and solubilized with 100 ㎕ of dimethyl sulfoxide (DMSO) The absorbance was measured at 540 nm by a spectrophotometer (Labsystem Multiskan, Labsystem, Helsinki, Finland).

4. Apoptosis analysis by flow cytometry

Ovarian cancer cells were cultured in 100mm culture dish. The next day, the cell was incubated in 0, 12, 24 and 48 h with 15 ㎛M curcumin. The cells were collected using tripsin/EDTA and the cell grown medium and washing PBS were collected together. All procedure was performed on ice. All of them were centrifuged 5min at 400g, 4℃, after that, the supernatant was discarded. The pellet was resuspended by phosphate buffered saline (PBS) and centrifuged (400g, 5 min, 4 ℃). To analyze the apoptosis, the cells were resuspended in 200㎕ staining buffer (Annexin V/PI staining kit, BD Biosciences) and stained by Annexin V-APC and PI.

5. Western Blotting

Curcumin treated cells were lysed with lysis buffer (0.1%

dichloroacetic acid (DCA), 1 mM phenylmethylsulfonyl Fluoride (PMSF), 10 μM protease inhibitor cocktail, 1 mM Na_3PO_4 and 1% Triton-X 100 were added to the premade 2 lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA)). The concentration of protein was decided by BCA protein assay kit (Thermo scientific). Protein was loaded 15 μg per well and transferred to nitrocellulose membrane (Bio-rad, Hercules, CA) for 1 h. The protein ladder was confirmed by Ponceau (0.1% Ponceau S in 5% acetic acid solution) and washed by 0.1% TBS-T for 3 times per 10 min. Membrane was blocked by 5% skim milk in 0.1% TBS-T and washed by 0.1% TBS-T for 3 times per 10 min. First antibody was dissolved in 1% BSA, 0.01% Sodium azide and 0.1% TBS-T. The membrane was incubated with first antibody for 4℃ and overnignt. Next day, the membrane was washed by 0.1% TBS-T for 3 times per 10 min and incubated with secondary antibody (1:5000) in 5% skim milk for 2 h at 25 ℃ . The membrane was developed using chemiluminescence detection kit (AbFrontier, Seoul, Korea).

6. Measurement of Ca^{2+}-flux by confocal microscopy and flow cytometry

Cells were seeded on 60 mm dishes for measurement of Ca^{2+} flux using flow cytometry. The next day, 15 μM curcumin were treated for 24 h and 48 h. The cells washed by PBS and collected after detached by trypsin-EDTA. The collected cell was centrifuged.

(400g, 5 min, 4 ℃) and replaced to FACS tubes (BD Biosciences, San Jose, CA) after resuspend by PBS. Centrifugation the solution (400g, 5 min, 4 ℃) and discarded the supernatant. The pellet resuspended by 200 ㎕ Fluo-4 staining solution (Invitrogen® Life Technologies) and incubated 40 min and analyzed by flow cytometry (FACS Calibur, BD Biosciences, NorthRyde, Australia). Cells were seeded on coverglass bottom dish (SPL Life sciences, Gyeonggi-do, South Korea) for measurement of Ca^{2+} flux by confocal microscopy (Zeiss LSM 510 Multiphoton Confocal Microscope, Carl Zeiss, Oberkochen, Germany). After 24 h from seeding the cells, the cells incubated with serum-free media for 24 h. The next day, the dishes washed by PBS and stained by 100 ㎕ Fluo-4 solution (Invitrogen® Life Technologies) for 40 min. The staining solution was suctioned and washed by PBS. Covered 1ml of 1 HBSS (with calcium chlolide and magnesium chlolide, Invitrogen® Life Technologies) the dishes and recorded the basal level for 6~10 s and the records were collected every two seconds. Fifteen micromol curcumin added the dished after set the basal level. Analysis recorded on 8 min for two seconds each. The data was analyzed using LSM 510 software.

7. RT-PCR

RNA was isolated by RNA isolation kit (Quiagen, Valencia, CA). The concentration of RNA was determined by Nano-drop(NanoDrop 2000, Thermo Scientific). The cDNAs were

amplified by PCR for 30 cycles (94℃ for 30 seconds, 57℃ for 30 seconds, and 72℃ for 30 seconds) using SYBR super mix (Bio-rad).
SERCA 1a forward primer 5' -AGCCCGTGTCACAGATCCAG-3' and reverse primer 5' -ATAAGCAGCTCAGCGCAGTCG-3', SERCA 1b forward primer 5' -TCGGAACTACCTAGAGGATCCAGA-3', and reverse primer 5' -TAAGCAGCTCAGCGCAGTCG-3', SERCA 2a forward primer 5' -AACGCCCTCAACAGCTTGTC-3' and reverse primer 5' -TCCAGTATTGCAGGTTCCAGGT-3', SERCA 2b forward primer and 5' -TCATCTTCCAGATCACACCGCT-3', reverse primer 5' - TCAAGACCAGAACATATCGC-3', SERCA 3 forward primer 5' -ATGCCCTCAACAGCGTCTCG-3' and reverse primer 5' -GCTCACTTCTGGCTCATTTCTTCGT-3', GAPDH forward primer and reverse primer

8. Isolation of Endoplasmic Reticulum

Isolation of endoplasmic reticulum was modified from Petros Bozidis et al.[27] Briefly, cells were grown on five to ten 150-mm plates and collected 3 days later. Collected cell was resuspended by 1 MTE buffer (270mM D-mannitol, 10mM Tris-base, 0.1mM EDTA, pH 7.4) with 1mM PMSF and sonicated at 30 watt on ice three times for 10sec each, separated by 10 s rest intervals. Lysed cells centrifuged (1400g, 10 min, 4 ℃) and collected supernatant. The collected supernatant was centrifuged 5,000g, 10 min, 4 ℃. After

centrifugation, the supernatant was replaced to new tube. For ER isolation, prepared discontinuous sucrose gradients before the isolation and keep it cold. Placed 2.0 M sucrose bottom of a 1489 mm Beckman polyallomer ultracentrifuge tube (Beckman Coulter, Brea, CA) and slowly layered 3 ml of 1.5 M sucrose (10 mM Tris-base, 0.1 mM EDTA, pH 7.6) onto the 2.0 M sucrose (10 mM Tris-base, 0.1 mM EDTA, pH 7.6) and added 3 ml of 1.3 M sucrose (10 mM Tris-base, 0.1 mM EDTA, pH 7.6) on top of the gradient. Two mililiter of the supernatant layered onto the top and placed 1 ml of ice-cold MTE buffer with 1 mM PMSF onto the top of layer. They were ultracentrifuged at 152,000g, 70 min, 4 ℃ by SW41 rotor (Beckman Coulter). After centrifugation, the large band at the interface of the 1.3 M sucrose gradient layer extracted by 20 G needle. The band diluted by 5 ml of MTE plus 1 mM PMSF buffer and centrifuged at 126,000g, 1 h, 4 ℃ (Beckman Coulter). Discard the supernatant and resuspended the pellet in 100 μl of 1MTE plus 1 mM PMSF buffer and stored at −70 ℃ after quickly frozen by liquid nitrogen. The concentration of protein was decided by BCA protein assay kit from Thermo scientific.

9. Measurement of Ca^{2+}-dependent ATPase Activity

Measurement of Ca^{2+}-dependent ATPase activity was modified from Yankun Li et al[28]. SERCA activity was measured using an NADH enzyme-coupled assay. The assay buffer contained 120 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 1 mM

dithiothreitol (DTT), 0.45 mM $CaCl_2$, 0.5 mM EGTA, 25 mM MOPS/KOH, 0.32 mM NADH, 5units/ml pyruvate kinase, 10 units/ml lactate degydrogenase, and 2 μM of the calcium ionophore A23187. The pH 7.0 was adjusted before addition of the enzyme. The microsome was incubated 10 min with 15 μM curcumin before adding assay buffer. Reaction was started by adding 10 μg of the ER to 200 μl of the assay buffer in 96 well plates. After 30 min incubation, the absorption detected at 355 nm using fluoremeter (Fluoroskan Ascent, Thermo scientific).

10. Primary culture of human ovarian surface epithelial cells

Human ovarian tissues were obtained from nonmalignant patients from Seoul national university hospital.(Seoul, South Korea) The tissues were gently washed with PBS and removed blood, blood vessel and stromal cell. Dispase (Gibco ® Life Technologies) was dissolved in 3’ distilled water about the 24units/ml stock concentration and stored at −20 ℃. The enzyme was dissolved in 9ml PBS before used and working concentration was 2.4unit/ml. The tissue surface was faced to bottom of Petri dish and covered with the enzyme to incubate overnight at 4 ℃. The next day, surface of the tissue was gently scrubbed and collected by dispase. Collected cells were diluted by PBS to 3 times the collected dispase and centrifuged. (500g, 4min, 25℃) After the supernatant removed, pellet was suspended by media which was 1 : 1 mixture of MCDB

105 (Gibco ® Life Technologies) and M199 (Sigma-Aldrich) supplymented 10% FBS and 1% penicillin/streptomycin and grown in 35 mm culture dish under 37 ℃ in a humidified atmosphere of 5% CO_2 (Thermo scientific). Human ovarian surface epithelial cells of passage 2 were used for RT-PCR and western blotting.

11. Isolation of peripheral blood mononuclear cell (PBMC) from buffy coat

Isolation of peripheral blood mononuclear cell (PBMC) from buffy coat was modified from Schnekenburger et al[29]. Briefly, the buffy coat was diluted with PBS. Ficoll-Plaque™-Premium (GE healthcare Bio-Sciences AB, Sweden, Uppasala) placed the bottom of new 15ml conical tube. The Ficoll-Plaque™- Premium volume was one-third amount of buffy coat. The diluted buffy coat was slowly layered onto the Ficoll-Plaque™- Premium. The conical tube was centrifuged 400g, 45min, 25 ℃. The plasma was discarded and the PBMC was replaced to the new tube. The replaced PBMC was washed using PBS by centrifugation at 180g, 15min, 25 ℃. The washing was replicated twice, after that, the supernatant was discarded and PBMC was diluted with RPMI 1640. The diluted PBMC was replaced to the FACS tubes (BD Biosciences) and incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 (Thermo scientific).

12. Transient transfection of SERCA2b

The ovarian cancer cells were transfected with human SERCA2b cDNA plasmid. This generous gift is from Dr Jonathan Lytton (Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada).

The cells were seeded on the 60mm dish. The next day, the human SERCA2b cDNA plasmid was transfected to the ovarian cells using Lipofectamin-LTX (Invitrogen® Life Technologies). After 24 h, the cells were replaced the dish for proper assay.

13. Statistical analysis

Results were presented as the mean ± SEM. The data were verified by student's t-test and ANOVA test. P-values < 0.05 was indicated as *.

RESULTS

Decrease of cell viability only in ovarian cancer cells after curcumin treatment

Three kinds of ovarian cancer cell lines which have different p53 status were used for analysis of cell viability after curcumin treatment (Fig. 1A). The cells were exposed to curcumin in different time (0, 24 and 48 h) and concentration (0, 10, 30 and 90 μM). After treatment of curcumin, the cell viability was measured by MTT assay. Cell viability was decreased in a concentrate- and a time-dependent manner in regardless of p53 status. IC_{50} values of MDAH2774 were 29.7 μM and 7 μM, SKOV3 were 3.5 μM and 1.3 μM, and PA1 were 18.6 μM and 6.5 μM for 24 and 48 h, respectively (Fig. 1B). The normal ovary surface epithelial cell (OSE) and peripheral blood mononuclear cell (PBMC) were as a normal control. The cell viability of PBMC did not decrease after exposure to 15 μM curcumin for 24 h. The cell viability of normal OSE was decreased after curcumin treatment, however, the percentage of live cell is much higher than of ovarian cancer cells (Fig. 1C).

Induction of apoptosis by curcumin in ovarian cancer cells

The cells were exposed to 15 μM curcumin during 0, 12, 24 and 48 h. After that, apoptosis was determined using flow cytometer

after PI/Annexin V-APC staining. Apoptosis population was significantly increased in a time-dependent manner (Fig. 2A). Apoptosis induction was confirmed by increase of PARP cleavage using Western blotting. After 24 h from curcumin treatment, PARP cleavage was significantly increased (Fig. 2B). However, the apoptotic cell death did not observed in OSE (Fig. 2C). Get together these results, curcumin induced apoptosis in ovarian cancer cell specific manner.

Increase of cytosolic Ca^{2+} after curcumin treatment

We measured cytosolic Ca^{2+} concentration to study whether curcumin affect Ca^{2+} concentration. DMSO was used as a negative control and 15 μM Thapsigargin (SERCA inhibitor) was used as a positive control. When the cells were exposed to DMSO, the cytosolic Ca^{2+} concentration was not increased. The cells were exposed to 15 μM Thapsigargin, the Ca^{2+} concentration was increased for a while. On the contrary to this, the Ca^{2+} concentration was elevated instantly and maintained in a high state after exposing of 15 μM curcumin (Fig. 3A). Also, cytosolic Ca^{2+} concentration of the ovarian cancer cells is steadily increased until 48 h after curcumin treatment. The PBMC was usued as normal control. The cytosolic Ca^{2+} concentration of PBMC was not changed after exposure of curcumin (Fig. 3B). Based on these data, we deduced that curcumin induced disruption of Ca^{2+} homeostasis in ovarian cancer cells.

Decrease of apoptosis after chelating of cytosolic Ca^{2+}

Five micromole of BAPTA was pre-treated to chelate cytosolic Ca^{2+} for a 1 h before curcumin treatment. After 12 h, the cells yielded for determine the apoptosis. The proportion of curcumin-induced apoptosis recovered after curcumin treatment with BAPTA-AM in MDAH 2774 and PA1. (Fig. 4A). The PARP cleavage was reduced in curcumin and BAPTA treated cells than in only curcumin treated cells, MDAH 2774 and PA1 (Fig. 4B). Therefore, we estimated that curcumin induced apoptosis was result from disruption of cytosolic Ca^{2+} homeostasis.

Elevation of SERCA2 protein in ovarian cancer more than in normal ovarian surface cell

In another paper reported that expression level of SERCA is much higher in cancer tissue than in normal tissue. Therefore, we studied about difference of SERCA expression between normal OSE and ovarian cancer cells. First, we examine the distinction of mRNA level of SERCA expression from OSE cell and cancer cell. SERCA1 mRNA was not expressed both OSE and ovarian cancer cells. The mRNA expression of SERCA2a and SERCA2b did not showed any difference between normal cell and cancer cells. But, SERCA3 mRNA was increased in MDAH2774 (Fig. 5A and 5B).

In protein level, SERCA2 expression of cancer cells was increased rather than OSE. However, SERCA3 was not expressed both normal cell and cancer cells (Fig. 5C and 5D).

Inhibition of SERCA activity by curcumin without change of protein level

We observed SERCA2 protein level after curcumin treatment. The protein expression of SERCA2 did not show any change after exposure of curcumin (Fig. 6A).

After isolation of endoplasmic reticulum(ER) from each ovarian cancer cells, we confirmed it by Western blotting. The isolated vesicle presented only ERp57 (ER marker protein). Neither cytochrome oxygenase 2 (COX2; mitochondria marker protein) nor pan-cadherin (marker protein of cell membrane) was not expressed in the isolated ER fraction (Fig. 6B). The SERCA activity was inhibited after curcumin treatment without any change of SERCA2 protein status (Fig. 6C).

Induction of apoptosis and cytosolic Ca^{2+} overload decline in SERCA2 overexpressed cells

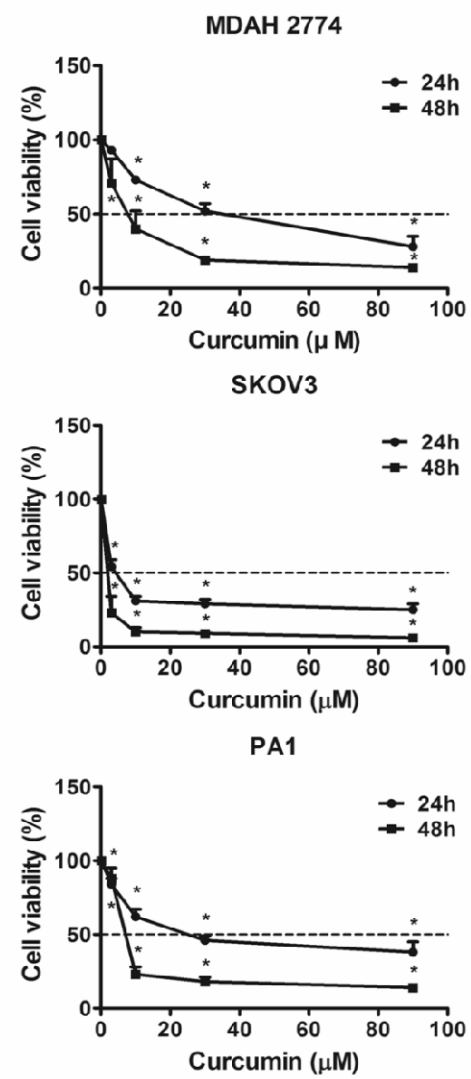
SERCA2b overexpressing cancer cells were evaluated whther curcumin induced Ca^{2+} disruption and apoptosis were linked to attenuation of SERCA activtiy. The PARP cleavage was attenuated in SERCA2b transfected cells (Fig. 7A). The increase late of cytosolic Ca^{2+} was decreased in trasnfected cells (Fig. 7B). Based on these data, we deduced that inhibition of SERCA activity by curcumin was linked to disruption of Ca^{2+} induced-apotosis.

FIGURES

(A)

Cell line	PA1	MDAH 2774	SKOV3
p53 status	Wild type	Mutant type	Null type

(B)



(C)

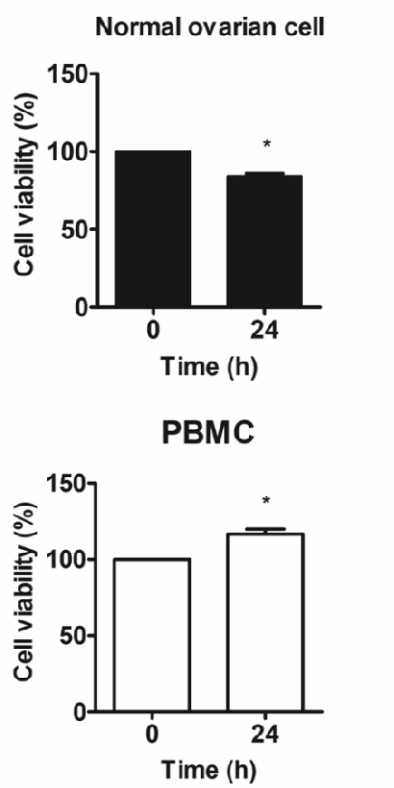
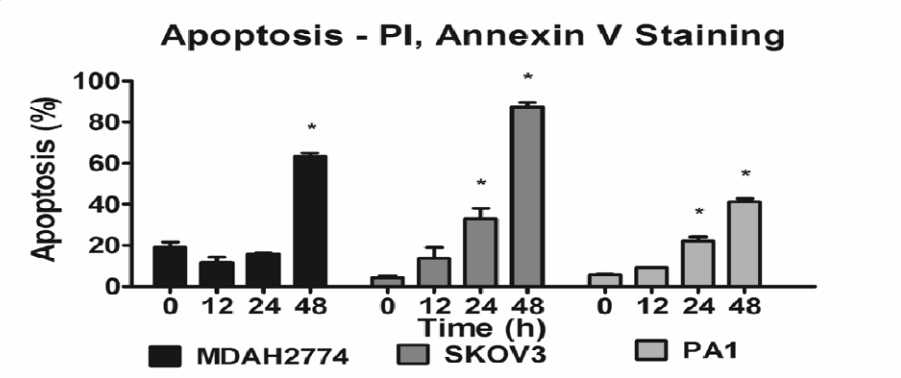


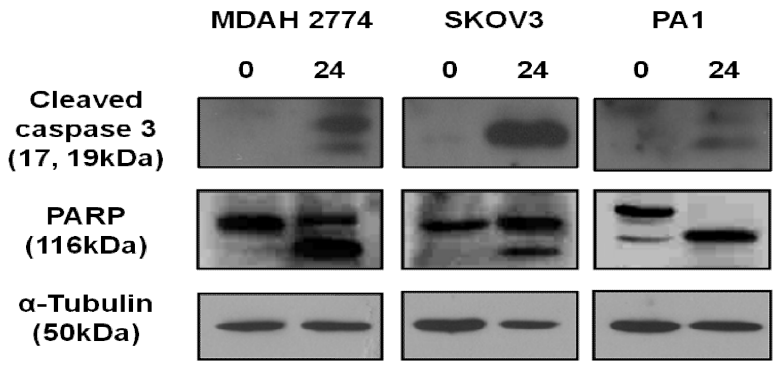
Figure 1 Suppression of cell viability by curcumin only in ovarian cancer cells, not in normal cells

(A) The p53 status of ovarian cancer cells; PA1 (p53 wild type), MDAH 2774 (p53 mutant type) and SKOV3 (p53 null type). (B) After treatment with 0, 10, 30, and 90 μM of curcumin for 24 and 48 h, cell viability was determined by MTT assay. The cell viability was decreased in a time- and concentration-dependent manner regardless of p53 status in ovarian cancer cells. (C) Normal ovarian surface epithelial cell (OSE) and PBMC were used as a normal control. The live cells of PBMC were counted using trypan blue staining after treatment of 15 μM curcumin and cell viability of normal OSE's was measured using MTT assay. The curcumin treatment did not affect the cell viability of normal control cells, such as OSE and PBMC. $*p<0.05$ vs. control

(A)


Apoptosis - PI, Annexin V Staining
Apoptosis (%)
100
80
60
40
20
0
0 12 24 48 0 12 24 48 0 12 24 48
Time (h)
MDAH2774
SKOV3
PA1

(B)


MDAH 2774
SKOV3
PA1
0 24 0 24 0 24
Cleaved caspase 3 (17, 19kDa)
PARP (116kDa)
α-Tubulin (50kDa)

(C)

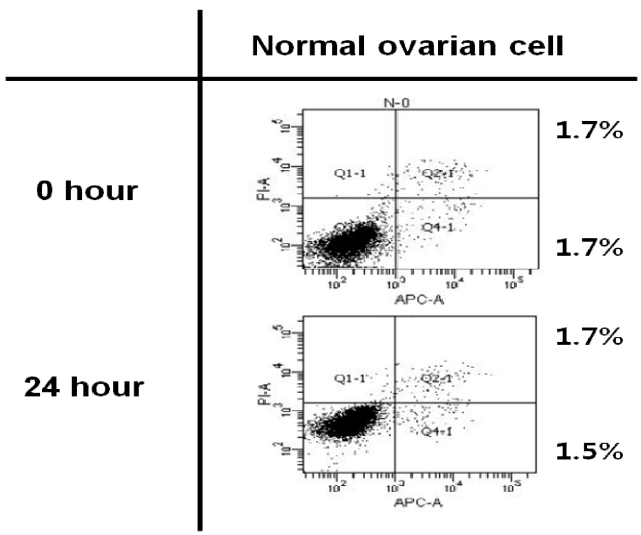

Normal ovarian cell
0 hour
24 hour
N-0
Q1-1
Q2-1
Q4-1
PI-A
APC-A
1.7%
1.7%
1.7%
1.5%

Figure 2 Measurement of apoptosis after treatment of curcumin

Cells were analyzed for determination of apoptosis after exposure to 15 μM curcumin. (A) The cells were exposed to curcumin in 0, 12, 24 and 48 h and analyzed using flow cytometry after PI/Annexin V-APC staining. Population of apoptosis was increased in a time-dependent manner. $*p<0.05$ vs. control (B) Cleavage form of PARP and caspse 3 was increased after curcumin treatment. (C) The apoptotic number of normal OSE's was not changed after curcumin treatment.

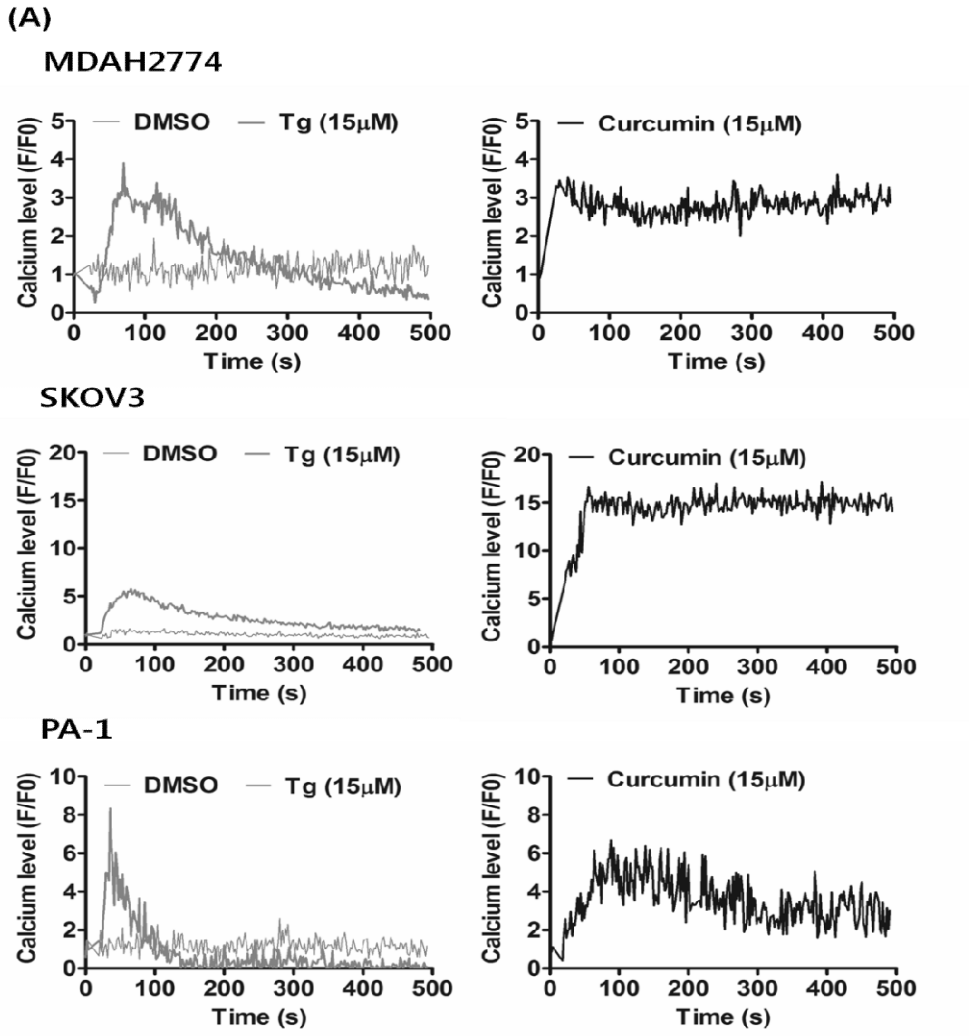
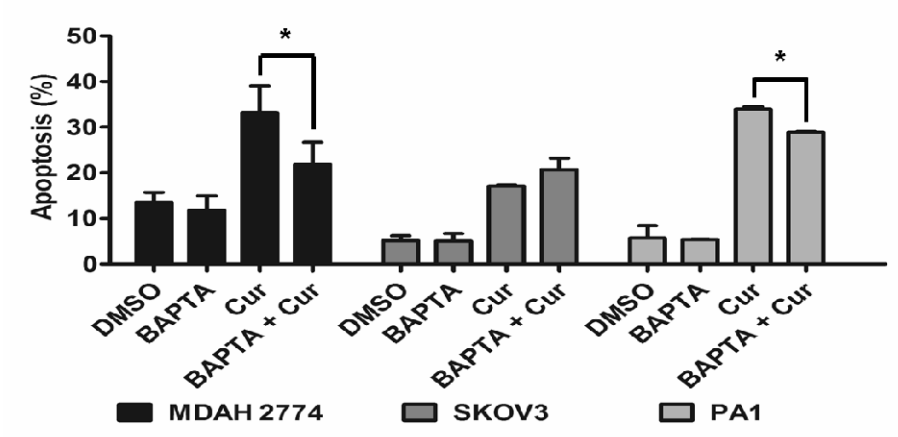

(A)
MDAH2774
DMSO
Tg (15μM)
Curcumin (15μM)
Calcium level (F/F0)
Time (s)
SKOV3
PA-1
(B)
Ca2+ level
Intensity (mean value)
Time (h)
PBMC
MDAH 2774
SKOV3
PA1

Figure 3 Increase of cytosolic Ca^{2+} level in curcumin-treated cell

Cytosolic Ca^{2+} concentration of ovarian cancer cells was observed after exposure to 15 μM curcumin. (A) Ca^{2+} flux was measured by confocal microscopy after Flou-4 staining. Cytosolic Ca^{2+} concentration was immediately increased after curcumin treatment. DMSO was used for the negative control, and 15 μM thapsigargin (Tg) for the positive control. (B) Persistently elevation of cytosolic Ca^{2+} level until 48 h after the treatment of 15 μM curcumin in ovarian cancer cells. However, the cytosolic Ca^{2+} level of PBMC was not increased after curcumin treatment. The Ca^{2+} concentration was measured by flow cytometer. $p<0.05$ vs. control

(A)


Apoptosis (%)
50
40
30
20
10
0
*
*
DMSO
BAPTA
Cur
BAPTA + Cur
DMSO
BAPTA
Cur
BAPTA + Cur
DMSO
BAPTA
Cur
BAPTA + Cur
MDAH 2774
SKOV3
PA1

(B)

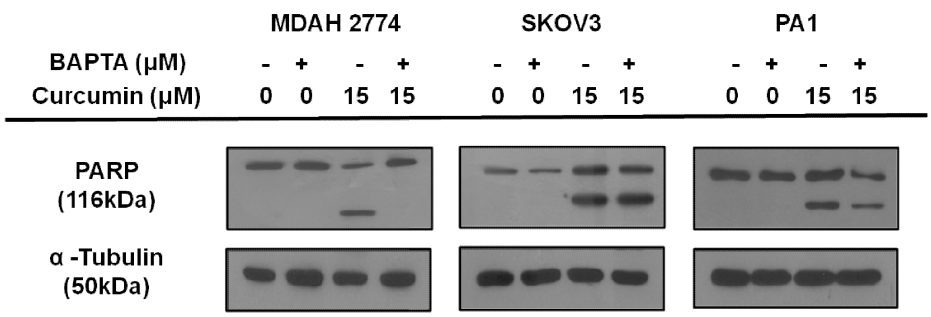

MDAH 2774
SKOV3
PA1
BAPTA (μM) - + - + - + - + - + - +
Curcumin (μM) 0 0 15 15 0 0 15 15 0 0 15 15
PARP
(116kDa)
α -Tubulin
(50kDa)

Figure 4 Suppression of curcumin-induced apoptosis with the addition of Ca^{2+} chelator

The cells were treated with 15 μM curcumin with or without BAPTA-AM (a Ca^{2+} chelator). (A) The apoptosis of cancer cells was measured using flow cytometry. The apoptotic cell number of curcumin and BAPTA treated cells was less than of curcumin-treated cells. $*p<0.05$ vs.control. (B) The cleaved-PARP was observed using Western blotting. The PARP cleavage was attenuated in the curcumin- and BAPTA-treated cells than in curcumin-treated cells.

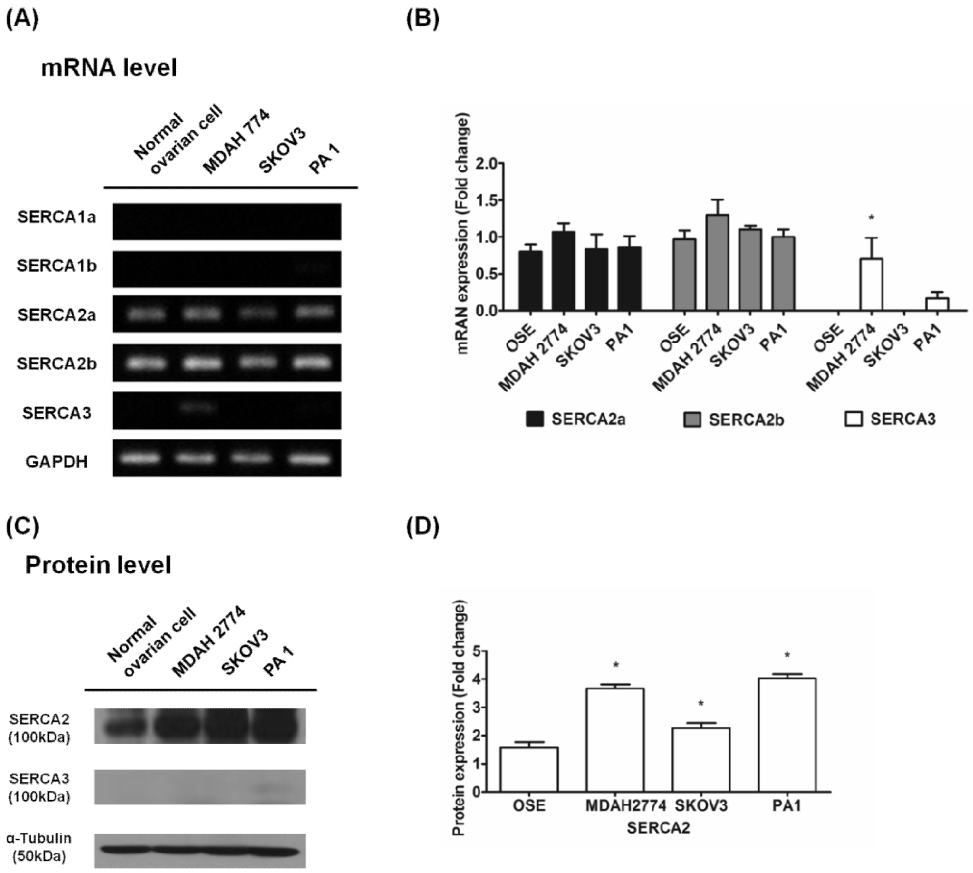

(A)
mRNA level
Normal ovarian cell
MDAH 774
SKOV3
PA 1
SERCA1a
SERCA1b
SERCA2a
SERCA2b
SERCA3
GAPDH
(B)
mRAN expression (Fold change)
2.0
1.5
1.0
0.5
0.0
OSE
MDAH 2774
SKOV3
PA1
SERCA2a
SERCA2b
SERCA3
(C)
Protein level
Normal ovarian cell
MDAH 2774
SKOV3
PA 1
SERCA2 (100kDa)
SERCA3 (100kDa)
α-Tubulin (50kDa)
(D)
Protein expression (Fold change)
5
4
3
2
1
0
OSE
MDAH2774
SKOV3
PA1
SERCA2

Figure 5 Basal level of SERCA mRNA and protein expression in normal OSE’ s and ovarian cancer cells

(A) SERCA2a and 2b mRNA were expressed in normal OSE’ s and ovarian cancer cells. SERCA3 mRNA was expressed only in MDAH 2774. (B) The densitometry graph was represented the expression level of SERCA2a, SERCA2b and SERCA3 mRNA. The expression of SERCA3 mRNA was increased only in MDAH 2774. $*p<0.05$ vs.control. (C)Protein expression of SERCA2 was higher in ovarian cancer cells than normal OSE’ s. SERCA3 was not detected in all cells at the protein level. (D) The densitometry graph was represented the expression level of SERCA2 protein. The expression of SERCA2 protein was elevated in all kinds of ovarian cancer cells. $*p<0.05$ vs.control.

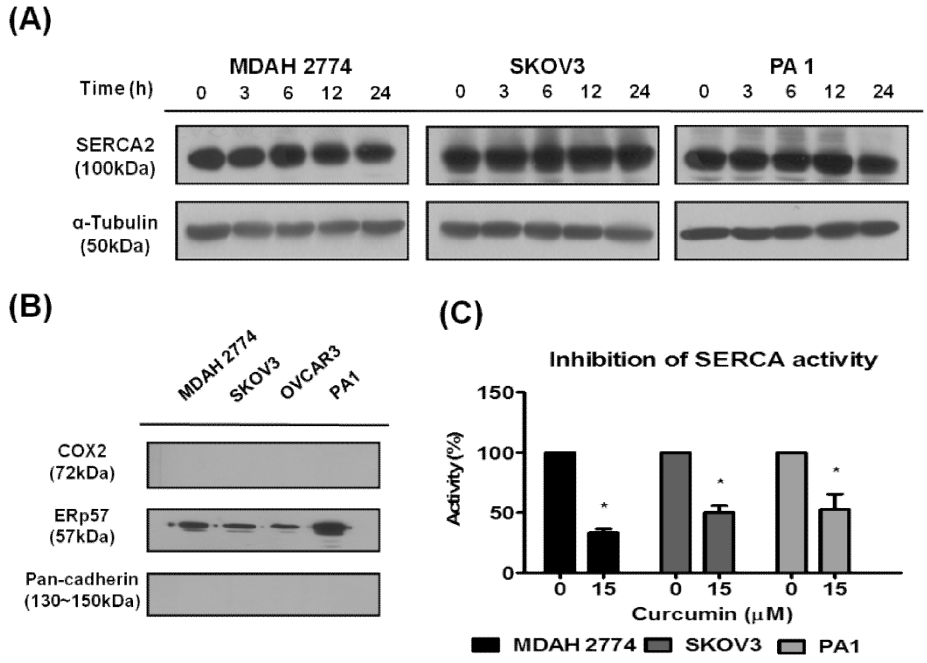
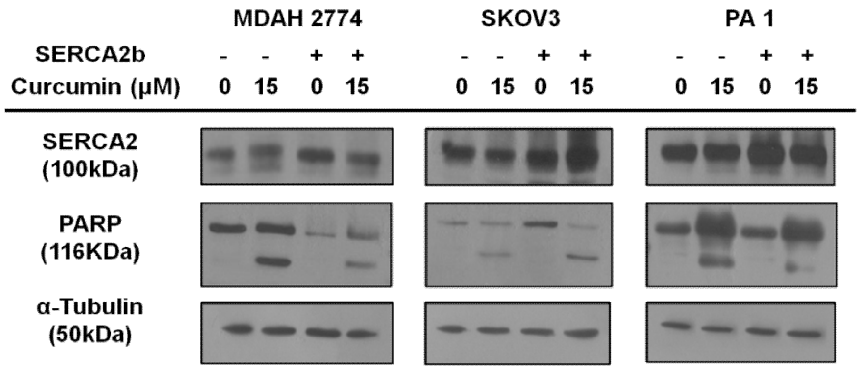

(A)
MDAH 2774
SKOV3
PA 1
Time (h)
0 3 6 12 24
SERCA2
(100kDa)
α-Tubulin
(50kDa)
(B)
MDAH 2774
SKOV3
OVCAR3
PA1
COX2
(72kDa)
ERp57
(57kDa)
Pan-cadherin
(130~150kDa)
(C)
Inhibition of SERCA activity
Activity (%)
150
100
50
0
0 15 0 15 0 15
Curcumin (μM)
MDAH 2774
SKOV3
PA1

Figure 6 No effect of curcumin on the expression of SERCA at the level of protein after curcumin treatment

Protein expression level of SERCA was measured after curcumin treatment. (A) Curcumin did not affect the protein level of SERCA2 in ovarian cancer cells. (B) Endoplasmic reticulum (ER) was isolated by sucrose density gradient in each ovarian cancer cell line. The isolated ER was confirmed by Western blotting using ERp57 (ER marker protein) antibody. Neither cytochrome oxygenase 2 (COX2; mitochondria marker protein) nor pan-cadherin (marker protein of cell membrane) was not expressed in the isolated ER fraction. (C) SERCA activity was determined by NADH enzyme-linked assay. Curcumin inhibited SERCA activity. $*p < 0.05$ vs. control

(A)


MDAH 2774
SKOV3
PA 1
SERCA2b
Curcumin (μM)
SERCA2 (100kDa)
PARP (116KDa)
α-Tubulin (50kDa)

(B)

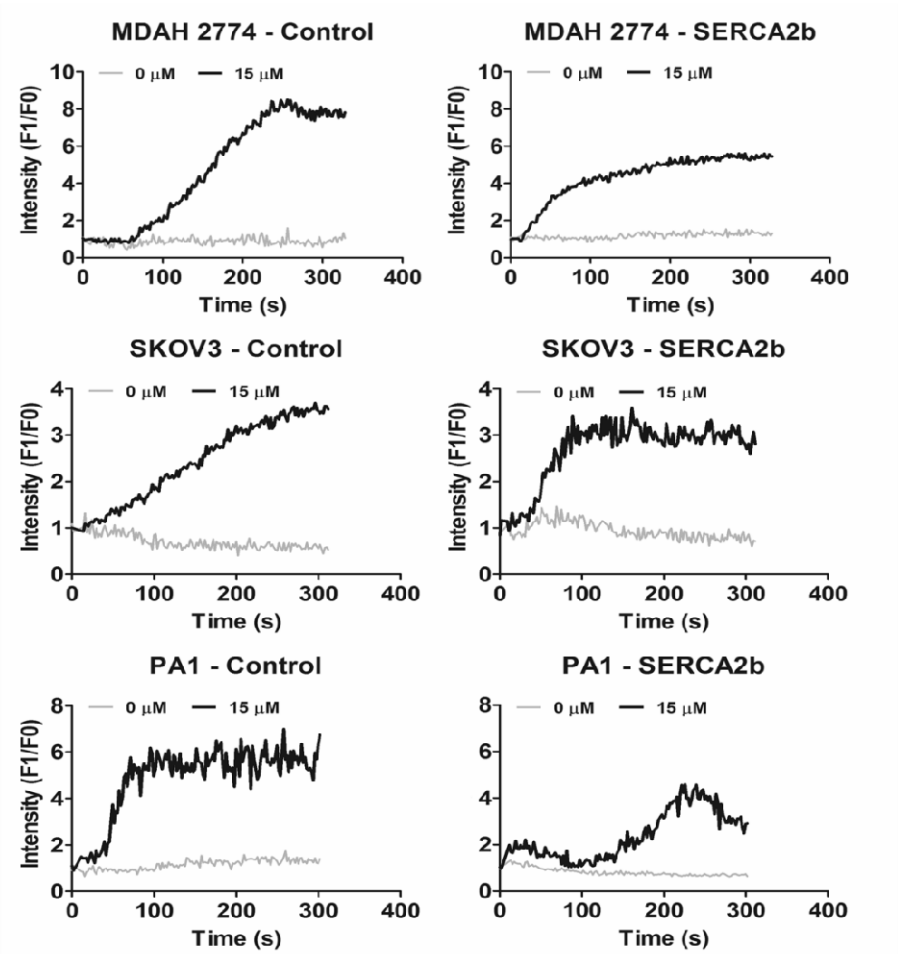

MDAH 2774 - Control
MDAH 2774 - SERCA2b
SKOV3 - Control
SKOV3 - SERCA2b
PA1 - Control
PA1 - SERCA2b
0 μM
15 μM
Intensity (F1/F0)
Time (s)

Figure 7 Decrease of apoptosis and cytosolic Ca^{2+}-elevation in SERCA2b gene transfected cells

The ovarian cancer cells were treated with curcumin following transfection with human SERCA2b cDNA plasmid. (A) SERCA2 was overexpressed in the SERCA2b-transfected cells. Curcumin did not increase the PARP cleavage (as an indicator for the apoptosis) in SEARCA2b-overexpressed cells. Tubulin-alpha was used as a loading control. All protein levels were assessed by Western blot. (B) The Ca^{2+} flux was measured by confocal microscopy. The cytosolic concentration of Ca^{2+} was lower in SEARCA2b-overexpressed cells than control ones.

DISCUSSION

The experimental data reveal that inhibition of SERCA activity by curcumin induces Ca^{2+} homeostasis disruption and it leads the ovarian cancer to the apoptotic cell death.

Curcumin is a non toxicity chemical with strong anticancer activity. In this report, we used the PBMC and normal surface epithelial ovarian cells (OSE) as a normal control to test the curcumin cytotocxicity. The cell viability of PBMC increased rather than decreased in presence of curcumin. Curcumin treated OSE showed the decrease of cell viability, but, the viability of OSE was much higher than of ovarian cancer cells in presence of 15 μM curcumin. Furthermore, the percent of apoptotic cell death in OSE was very lower than in ovarian cancer cells. Curcumin does not show the toxicity in normal cells[30] including PBMC[31],[32], however, the effect of curcumin on OSE does not reported. The different cytotoxicity of curcumin is on account of the distinction of cellular uptake. Absorption of curcumin in cancer cell is much higher compared to normal cell. Curcumin is a lipophilic compound and interacts with cell membrane.[33] It is because of the difference of composition of cell membrane, membrane structure and cell size.[34,35] Similarly with the in vitro studies, curcumin shows the less toxicity in phase I clinical study[36]. In contrast to the effect of curcumin on normal cell, curcumin induced apoptosis in ovarian cancer cell lines in regardless of p53 status. p53 is the most frequently mutated gene which is altered about 96% in ovarian cancer[37]. Altered p53 plays

an important role in tumor progresseion[38,39] and in chemo-resistant[40]. However, curcumin induced-apoptosis independent of p53 status in various cancer cells[41,42] including ovarian cancer[13,43] and sensitization to apoptosis in chemo- and in radio-resistant ovarian cancer cell line[44].

Increased Ca^{2+} concentration of extracellular[45] and intracellular[46] is strongly related with ovarian cancer cell proliferation. However, amplified cytosolic Ca^{2+} is strongly linked with the cytotoxicity[47]. Increase of the large amount of cytosolic Ca^{2+} precipitates accumulation of Ca^{2+} in mitochondria and motivates the apoptosis[48]. In our results, the cytosolic Ca^{2+} was persistently elevated in ovarian cancer cells after exposure of 15 μM curcumin. After that, we proofed curcumin induced cytosolic Ca^{2+} elevation was involved with apoptosis using a Ca^{2+} chelator (BAPTA). Similarly with our data, other cancer cells including ovarian, colorectal and lung cancer with curcumin and BAPTA show the reduction of apoptotic cell death[49,13,50].

The Ca^{2+}-regulated pump as a SERCA is altered their expression in cancer to escape from apoptosis[7, 51] and for a proliferation[4,52]. SERCA has three kinds of subfamily including SERCA1, SERCA2 and SERCA3. The SERCA expression is depending on the cell type and the cytosolic Ca^{2+} concentration[4]. Our data was indicated that the protein level of SERCA2 expression was elevated in ovarian cancer cells rather than in OSE. However, the protein of SERCA1 and SERCA3 was not expressed both OSE and ovarian cancer cells. In the clinical study, mRNA and protein expression of SERCA2 are

overexpressed in colorectal cancer tissues than in normal colon tissues[8]. Also in ovarian cancer tissue, level of SERCA2 mRNA is highly expressed than in normal ovarian epithelial tissue[26] and in ovarian cancer cell line A2780, which is acquired platinum-resistant cell, is expressed SERCA2 mRNA higher than in control cell.[53] Thus, overexpressed SERCA plays an important role in tumorigenesis and chemo-resistant.

The SERCA activity was partially inhibited after curcumin treatment without any change of SERCA2 protein status. Curcumin inhibits SERCA activity in various cell types through binding with hinge site of SERCA and inhibits transformation of SERCA[16]. However, the curcumin concentration of SERCA inhibition in normal cell is much higher than our concentration[16].

Overall, this study demonstrated that curcumin induced apoptosis in ovarian cancer cell lines but there was no cytotoxicity on normal ovarian cell and PBMC. Inhibition of SERCA activity by curcumin evokes the elevation of cytosolic Ca^{2+} concentration and finally promotes apoptosis. These data indicated that curcumin is an effective chemotherapeutic agent in ovarian cancer without any toxicity in normal cell.

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국문 초록

Sarco/endoplasmic Reticulum Calcium ATPase (SERCA)는 소세포체 표면에 존재하는 칼슘 펌프로, 세포질에 존재하는 칼슘을 소포체내로 이동시킴으로써 세포 내 칼슘농도 항상성을 조절한다. 또한 난소암에서 SERCA2의 mRNA 발현이 증가되어있음이 보고된바 있다. 본 연구에서는 curcumin에 의해 억제된 SERCA의 활성억제를 통한 칼슘농도항상성의 저해가 난소암 세포주 특이적으로 자가세포사멸을 일으키는지에 대해 연구하였다. 난소암 세포주에 curcumin 처리시, 농도와 처리시간에 유의하게 세포성장이 감소하였으며, 자가세포사멸 또한 처리시간에 유의하게 증가하였다. 그러나 정상난소표피세포와 말초혈액단핵구에서는 curcumin을 처리하여도 이러한 현상이 보이지 않았다. curcumin 처리시, 세포질 내 칼슘농도는 48시간까지 지속적으로 증가하였으며, 칼슘제거제인 BAPTA-AM와 curcumin을 동시 처리하였을 때, 세포사멸이 회복되는 것을 통해, curcumin에 의한 세포질 내 칼슘농도 증가가 curcumin에 의해 유도되는 자가세포사멸에 중요한 역할을 하는 것을 알 수 있었다. 정상난소표피세포와 난소암세포주의 SERCA발현의 차이를 RT-PCR과 Western blotting을 통해 알아본 결과, SERCA1, SERCA2 and SERCA3의 mRNA 발현은 차이가 없었으나 난소암세포주에서 SERCA2 단백질의 발현이 더 높게 나타나는 것을 확인하였다. 난소암세포주에 curcumin을 처리하였을때, SERCA2의 단백질 발현에는 차이가 없었으나, SERCA의 활성이 감소됨을 확인하였다. SERCA2b의 과발현을 통해, SERCA2의 과발현시, 자가세포사멸이 회복되었고 세포질 내의 칼슘 증가도 대조군에 비해 적은 것을 확인하였다. 이를 통해, SERCA가 난소암세포사멸에 중요한 역할을 하는 것을 밝혔다. 본 연구의 결과를 종합해보면, curcumin에 의한 SERCA의 활성저해는 세포의 칼슘 항상

성의 저해를 유도하고, 이를 통해 자가세포사멸이 일어난다고 결론 지을 수 있다.