이학 석사 학위논문

Reactive oxygen species (ROS)-mediated Akt inhibition by genistein induces autophagic cell death in ovarian cancer cells

난소암 세포주에서 Genistein에 의해 유도되는 활성산소에 의한 Akt 저해와 자가포식의 항암작용과 기전 연구

2014 년 2 월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

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지도교수 송 용 상

이 논문을 이학 석사 학위논문으로 제출함

2013 년 10 월

서울대학교 대학원

협동과정 종양생물학 전공

이 현 주

이현주의 이학 석사 학위논문을 인준함

2013 년 12 월

위 원 장 (인)

부위원장 (인)

위 원 (인)

Reactive oxygen species (ROS)–mediated Akt inhibition by genistein induces autophagic cell death in ovarian cancer cells

by

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A thesis submitted in partial fulfillment of the
requirements for the Degree of Master of Science
to faculty of college of Medicine
in Seoul National University

December 2013

Approved by Thesis Committee:

Professor _____Chairman
Professor _____Vice chairman
Professor _____

ABSTRACT

Cancer cells are vulnerable to reactive oxygen species (ROS) due to redox imbalance. Recently, genistein, a polyphenolic compound, has shown to have pro-oxidant activity, causing cell death in several types of malignancies, including breast, prostate and hematologic cancers. Here, we show that in ovarian cancer cells genistein invokes the cell death through autophagy and the generation of ROS. As a matter of fact, genistein increases the level of ROS giving rise to apoptosis and autophagy. In addition, pretreatment with the autophagy inhibitor, 3-methyladenine (3-MA), suppressed genistein-mediated cell death in ovarian cancer cells. These results suggest that autophagy is involved in genistein-mediated cell death. Interestingly, genistein-mediated cell death was restored by ROS scavengers, such as N-acetyl-L-cysteine (NAC) and Trolox, suggesting an essential role of intrinsic ROS in genistein-induced autophagic cell death. Moreover, genistein stimulates the inhibition of the AKT/mTOR signaling pathway through a ROS-mediated mechanism. Taken together, our study suggests that genistein-mediated cell death through inhibition of the AKT/mTOR signaling pathway and ROS-induced autophagy is important for cancer cell killing.

Keywords: ROS, Autophagy, Genistein, Ovarian cancer, Apoptosis

Student Number: 2010-23728

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LIST OF ABBREVIATIONS

ROS : reactive oxygen species

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

DCF-DA : 2', 7'-Dichlorofluorescin diacetate

AO : Acridine orange

AVO : Acidic vesicular organelle

MDC : Dansylcadaverine

LC3 : light-chain 3

3-MA : 3-methyladenine

NAC : N-acetyl-cysteine

INTRODUCTION

Ovarian cancer is the most lethal gynecological malignancy in woman worldwide. Generally ovarian cancer is asymptomatic and, therefore results in late diagnosis, so that a large number of patients are at the advanced stage III/IV. Patients usually respond to surgery and primary chemotherapy. However, most patients develop recurrence and acquire resistance to conventional chemotherapy (1, 2). Over the last few decades, the 5-year survival rate for patients has marginally increased, but the overall cure rate remains roughly 30% (3). Therefore, novel potent therapeutic strategies are needed to treat ovarian cancer.

Cancer cells generally have a higher basal ROS level than normal cells, because of their increased proliferative activity (4, 5). Thus, cancer cells are more vulnerable to damage by additional ROS insults. Excessive ROS level induces oxidative stress and, subsequently leading to macromolecule damage, autophagy and cell death (6-8).

Autophagy is an emerging key regulator of cell death pathways, and has become a promising issue in cancer research (9). Autophagy is a highly conserved, lysosomal process that degrades long-living proteins and damaged organelles. Autophagy has a protective role in cancer cells in conditions of nutrient deprivation or metabolic stress. But also, autophagy has been demonstrated as the cell death mechanism in various types of cancer in response to anticancer therapies (10-12). Thus autophagy has the dual role,

oncogenic or tumor suppressive.

The Akt/mTOR signaling pathway has been shown to be associated with the chemoresistance in ovarian cancer study (1, 3). Akt (protein kinase B), a serine/threonine kinase, is the regulator of a cell survival function in response to growth factor stimulations (13). A mammalian target of rapamycin (mTOR) is also, essential regulator of cell survival process, inhibiting the autophagy (14). Akt and the mTOR signaling pathway could be inactivated by ROS generation, followed by cell death (15-17).

Most of anticancer agents induce ROS generation. Genistein, a natural flavonoid, in soy products has been thought to be the antioxidant (18). Recently, several studies have reported that genistein induced oxidative stress (19-21). However, the anticancer mechanism of ROS by genistein is not fully understood.

The present study explored the mechanism involved in the generation of ROS and autophagy by genistein in ovarian cancer cells. Our data provide the first evidence that genistein induced autophagic cell death in ovarian cancer cells through the ROS -mediated inhibition of Akt/mTOR signaling pathway.

MATERIALS AND METHODS

1. Reagents and Antibodies

Genistein (5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) was purchased from Sigma-Aldrich. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma-Aldrich), Annexin V-FITC Apoptosis Detection kit I(BD Pharmigen), Acridine orange (AO), Dansylcadaverine (MDC), 3-methyladenine (3-MA), N-Acetyl-L-cysteine (NAC) and Trolox, 2',7'-Dichlorofluorescin diacetate (DCF-DA), were from Sigma-Aldrich. The antibodies against LC3B, Beclin 1, P-p70S6K, p70S6K, P-4EBP-1, 4EBP were from Cell Signaling Technology. The P-Akt and total Akt were obtained from Santa Cruz Biotechnology. The α-Tubulin antibody (used as loading control) was purchased from the Sigma-Aldrich Chemical Company.

2. Cell lines and culture

Cells (PA-1, MDAH 2774 and OVCAR-3) were purchased from the American Type Culture Collection (Rockville, MD). PA-1 has been isolated from ovarian teracarcinoma and MDAH 2774 and OVCAR-3 have been isolated from adenocarcinoma of the human ovary. Cells (MDAH 2774 and OVCAR3) were cultured in RPMI 1640 medium (Hyclone Laboratories, Inc., Logan, UT) and PA-1 was cultured in MEM (Gibco-BRL, Gaithersberg, MD), supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified atmosphere at 37℃ in a 5% CO2 .

3. Viability assay

Ovarian cancer cells were seeded on 96-well plates with genistein at various concentrations (5, 10, 25, 50, 100 μM) to a final volume of 100 μl per well. Cells were incubated at 37℃ with 50 μl MTT solution (2 mg/ml in PBS) for 3 hours. Then cells were solubilized with 100 μl DMSO for 30 min. The optical density was measured at 540 nm with an ELISA reader.

4. Apoptosis assay

Cells were seeded into 60-mm culture dish with 4 ml and then treated with different concentrations of genistein (25, 50, 100 μM) for 24 hours. Genistein-treated cells were trypsinized, washed with cold PBS twice and re-suspended 1x Annexin V binding buffer. FITC-conjugated Annexin V and propidium iodide (PI) were added and incubated for 30 mins at room temperature in the dark. The samples were analyzed by flow cytometry, using the Annexin V-FITC Apoptosis Detection kit I (BD PharMingen).

5. Western blotting

Genistein-treated cells were harvested, washed and resuspended in lysis buffer for 20 min on ice. The cell lysates were centrifuged at 13,000 rpm for 20 min, after which clear supernatant was collected. The protein concentration was measured using the BCA Protein Assay Kit (Thermo SCIENTIFIC). Equal amounts of sample were loaded on SDS-PAGE (polyacrylamide gel electrophoresis), transferred to nitrocellulose membrane and detected with

primary and secondary antibodies. Detection was performed using chemiluminescence (ECL) system.

6. Immunofluorescence Confocal microscopy analysis

Cells were incubated with 100 μM genistein on Confocal Dish (Cover glass-Bottom Dish) for 24 hours. To detect acidic vesicular organelles, Acridine orange (AO) was used 1 μg/ml for 15 min at 37℃ (22). Stained cells were detected by fluorescence microscopy. Monodansylcadaverine (MDC) has been used for labeling autophagic vacuoles. Cells were stained with 50μM MDC at 37℃ for 30 min. After incubation, cells were fixed with 4% paraformaldehyde, washed two times with PBS and then analyzed by confocal microscopy (23).

7. Measurement of intracellular ROS generation

ROS generation was detected using DCF-DA staining by flow cytometry. Cells were treated with 100 μM genistein for 6, 24 hours and incubated with 25 μM DCF-DA for 30 min in the dark at 37℃. The treated cells were then harvested and washed twice with PBS. The cells were suspended in PBS and analyzed using flow cytometry analysis.

8. Stable transfection of activated Akt-tag cDNA

Human constitutively active Akt containing plasmid (CA-Akt) was generously given by Prof. Kangyeol Choi (Yeonsei University, Korea) (24). Transfection

of PA-1 cells was carried out using Mirus reagent (Mirus Bio LLC, Madison, USA), according to the manufacturer's instructions.

9. Isolation and culture of human ovarian surface epithelial (OSE) Cells

For normal ovarian surface epithelial cells, we followed the IRB approved protocol(c-1307-008-502). The normal ovarian surface epithelial(OSE) cells from fresh normal ovaries of patients were isolated by using Dispase (Gibco-BRL, Gaithersberg, MD) (25). Isolated OSE cells were incubated at 37℃ in complete growth medium (MCDB105 /M199 contained with 10% FBS and 1% P/S). All experiments using normal OSE were examined at culture passages 3-4.

10. Statistical analysis

Results were presented as the mean ± standard deviation (SD) or standard error of the mean (SEM). The data were verified by student's t-test and ANOVA test. P-values < 0.05, 0.01, 0.001 were indicated as *, **, ***, respectively.

RESULTS

Genistein decreases cell viability in ovarian cancer cells.

We initially evaluated the effect of genistein on ovarian cancer cell viability with the MTT assay. The ovarian cancer cell lines [PA-1(p53 wild type), MDAH2774 and OVCAR-3(mutant)] were treated with various concentrations of genistein (5-100 μM) for 24 hours. Genistein inhibited ovarian cancer cell growth in a concentration-dependent manner (Fig. 1). These results demonstrate that genistein induced cell death in ovarian cancer cells, unrelated with the p53 status.

Genistein treatment leads to the generation of intracellular ROS in ovarian cancer cells.

To determine the effect of genistein on ROS generation in ovarian cancer cells, DCF-DA (2',7'-dichloroflurescein diacetate), a specific ROS-detecting fluorescent dye, was used. The results showed that Genistein increased ROS levels in a dose- and time-dependent manner in all ovarian cancer cells, whereas in normal ovarian epithelial cells no change was observed after treatment with genistein. The PA-1 cell line showed markedly increased ROS levels as compared to the other two cell lines. We will further focus on this particular cell line in the remaining part of this paper.

Genistein induces apoptosis in ovarian cancer cells.

Genistein has been reported to induce anti-cancer activity through both apoptosis and autophagy (26, 27). In order to examine whether genistein induces apoptosis, we measured apoptotic cells using Annexin V/PI staining by FACS. Genistein-treated cells showed increased apoptotic cell death in a dose-dependent manner, but no significant change was observed in normal ovarian epithelial cells. In addition, cleaved PARP, a nuclear marker of apoptosis, was detected in PA-1 cells upon treatment with genistein. These data indicates that genistein induced apoptosis in ovarian cancer cells. Pre-treatment with z-VAD-fmk, a pan caspase inhibitor, had partial effects on the cell viability of genistein-treated cells, as analyzed by the MTT assay (Figure 3C).

Genistein induces autophagy in ovarian cancer cells.

Autophagy is the process of sequestering cytoplasmic components in a membrane vacuole, called autophagosomes (28). Recently, autophagy has been shown to trigger and mediate type II programmed cell death (8, 29). To investigate whether genistein can also induce autophagy in ovarian cancer cells, we analyzed acidic vesicular organelle (AVO) formation in genistein–treated cells, using AO (acridine orange) staining by FACS assay (Figure 4A) and fluorescence microscopy (Figure 4B)(22). Genistein induced the accumulation of acidic vesicles in the cytoplasm of ovarian cancer cell but no change was observed in normal ovarian epithelial cells (Figure 4A). The

MDC (Monodansylcadaverine) staining was additionally used to detect the autophagic vacuoles, because MDC can accumulate in late autophagosomes or autolysosomes in acidic vacuoles (30). In MDC staining, the bright blue dot, which indicates autophagic vacuoles, was increased in the cytoplasm (Figure 4C). The microtubule-associated protein 1 light-chain 3 (LC3) is essential for autophagy and the conversion of LC3-I to LC3-II is associated with the autophagosome membrane (31, 32). The conversion of LC3-I to LC3-II and the expression of beclin 1 can be used as a specific autophagy marker. LC3-I to LC3-II conversion and beclin 1 expression were evaluated by Western blot analysis (Fig. 4D). The result showed an increased level of LC3-I to LC3-II conversion and an increase of beclin 1 after treatment with genistein for 24 hours (Figure 4D). All these results indicated that genistein induced autophagy in ovarian cancer cells.

Genistein mediates autophagic cell death in ovarian cancer cells.

To confirm the role of genistein-induced autophagy in ovarian cancer cells, we used an autophagy-specific inhibitor, 3-MA (33). The effect of 3-MA on the induction of apoptosis was determined by Annexin V/PI staining analysis. As shown in Figure 5D, after genistein treatment in the presence of 3-MA, the percentage of apoptotic cells was considerably decreased, as compared to genistein treatment alone. This result indicates that genistein-triggered autophagy is a cell death process.

Z-VAD inhibits genistein-induced apoptosis, but not autophagy.

To determine the relationship between apoptosis and autophagy, induced by genistein in ovarian cancer cells, the pan-caspase inhibitor Z-VAD-fmk was used. The effect of Z-VAD-fmk on the apoptosis was determined by Annexin V/PI staining. Genistein treatment in the presence of Z-VAD-fmk significantly decreased the number of apoptotic cells, as compared to genistein treatment alone at 24 hours (Figure 5A). We then examined the autophagic induction after gensitein treatment in the presence of Z-VAD-fmk. The rate of autophagic cells in treatment with combination genistein and Z-VAD-fmk did not significantly change in ovarian cancer cells (Figure 5B). These results indicate that Z-VAD-fmk suppressed genistein-induced apoptosis, but not autophagy and suggests that genistein-induced autophagy may be located upstream of apoptosis.

Accumulation of ROS by genistein results in decreased cell viability and increased autophagy.

A recent study showed that ROS can regulate the signaling pathway to induce cell death and autophagy in various cancer cells (34-36). First, we checked whether pre-treatment with NAC, a well known ROS scavenger, could reduce ROS generation, using DCF-DA staining and FACS analysis. Pretreatment with NAC resulted in a significant decrease in the generation of

ROS, compared to genistein alone. To validate the role of ROS in ovarian cancer cell viability, we measured the effect of the ROS scavenger NAC or Trolox on cell growth inhibition by genistein. Ovarian cancer cells were treated with genistein in the presence or absence of NAC or Trolox for 24 hours and cell viability was measured by the MTT assay. As shown in Figure 6A, NAC or Trolox partly restored cell growth inhibition by genistein, suggesting that ROS generation plays an important role in the growth inhibition of genistein. Whether ROS plays a role in the induction of autophagy by genistein in ovarian cancer cells was examined by measuring AVO formation and conversion of LC3 I to LC3 II using Western blot. Ovarian cancer cells were pretreated with NAC for 1 hour, before treatment with genistein for 24 hours and staining with acridine orange. In Figure 6C, it can be seen that NAC significantly decreased the percentage of AVOs formation by genistein. As shown Figure 6B, the conversion of LC3 I to LC3-II, induced by genistein, was inhibited by pre-treatment with NAC. Taken together, these results suggest that genistein-induced increase of ROS production contribute to decreased cell viability and increased autophagy.

NAC blocks the inhibition of Akt/mTOR signaling molecules by genistein.

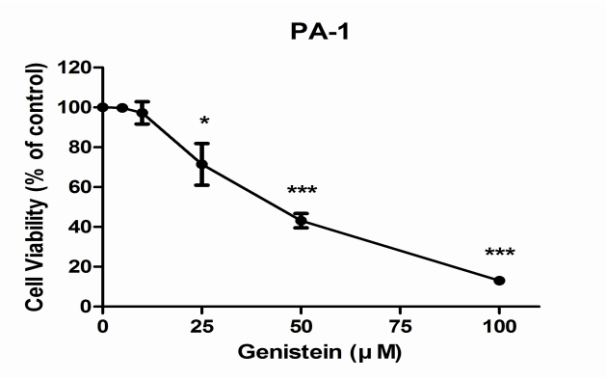
Akt/mTOR signaling is an important target responding to ROS stress (15, 16, 37). Therefore, we investigated whether genistein treatment has an effect on Akt/mTOR signaling proteins. Ovarian cancer cells were treated with

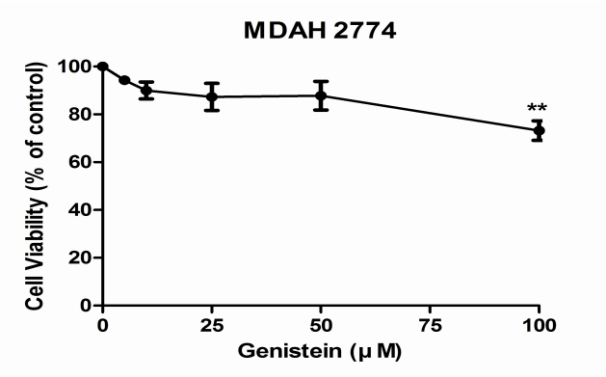
genistein (25, 50, 100 μM) for 24 hours. As shown in Figure 7A, phosphorylation of Akt, and p70S6K and 4E-BP1, downstream molecules of mTOR, were decreased in a dose-dependent manner following genistein treatment. Next, we determined whether genistein-mediated ROS generation is involved in the inhibition of these signaling proteins. Pretreatment with NAC suppressed the inhibition of Akt, and p70S6K and 4E-BP1 by genistein in ovarian cancer cells (Figure 7B). This result suggests that ROS may be involved in genistein-mediated regulation of the Akt/mTOR signaling pathway in ovarian cancer cells.

Akt signaling is required for genistein-induced autophagy.

To elucidate the role of Akt signaling pathway in genistein-induced autophagy, we transfected PA-1 cells with a constitutively active Akt (CA-Akt) (Figure 8A). Akt- overexpressing cells were treated with genistein, and the induction of autophagy was measured. As shown in Figure 8B and 8C, autophagic vesicles were decreased significantly in CA-Akt–overexpressing cells, as compared to control cells in the presence of genistein. Active CA-Akt-transfected cells were significantly more resistant to genistein-induced autophagy than control cells. Therefore, this suggests that genistein induces autophagic cell death in ovarian cancer cells by suppression of the Akt signaling pathway.

FIGURES


PA-1
Cell Viability (% of control)
120
100
80
60
40
20
0
*
***
***
0
25
50
75
100
Genistein (μ M)


MDAH 2774
Cell Viability (% of control)
100
80
60
40
20
0
**
0
25
50
75
100
Genistein (μ M)

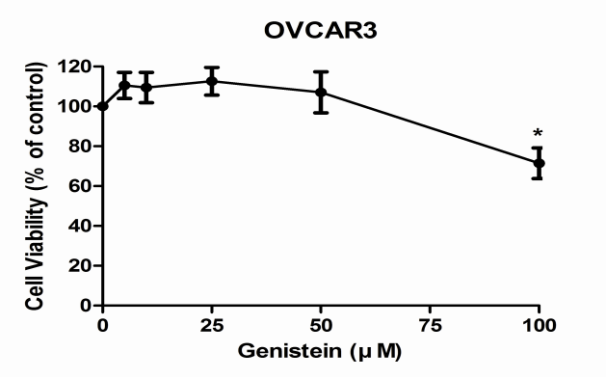
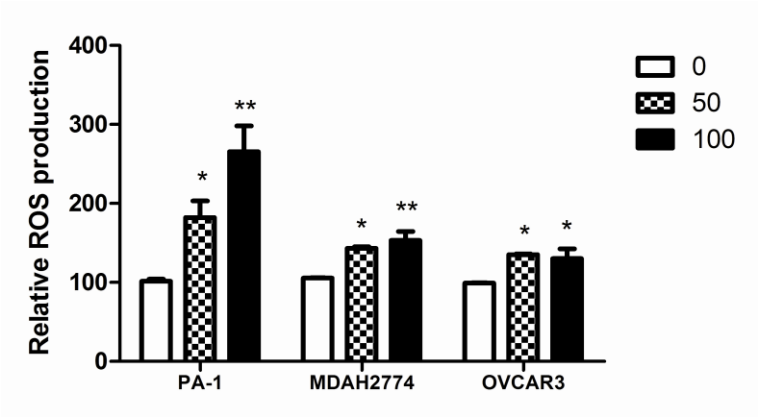

OVCAR3
Cell Viability (% of control)
120
100
80
60
40
20
0
*
0
25
50
75
100
Genistein (μ M)

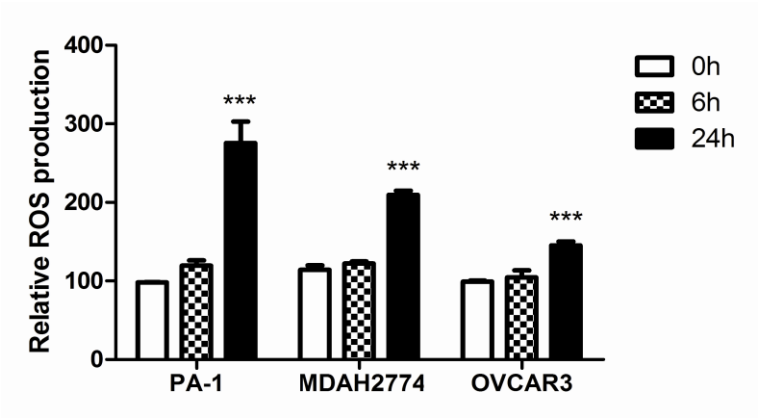
Figure 1. Decreased cell viability by genistein in ovarian cancer cells

Ovarian cancer cell lines, PA-1, MDAH2774 and OVCAR-3 were plated onto 96 well plates. Cells were treated at the various concentrations (0 – 100 μM) of genistein for 24 hours. The cell viability was measured by the MTT assay. Data represent the mean ± SD of three independent experiments. **P<0.05, **P<0.01 and ***P<0.001* vs. control

A


Relative ROS production
400
300
200
100
0
*
**
*
**
*
*
PA-1
MDAH2774
OVCAR3
0
50
100

B


Relative ROS production
400
300
200
100
0
***
***
***
PA-1
MDAH2774
OVCAR3
0h
6h
24h

C

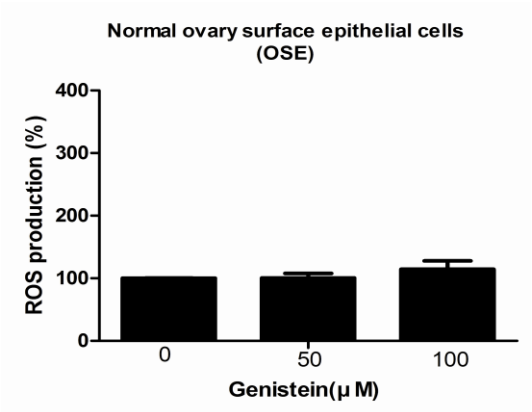
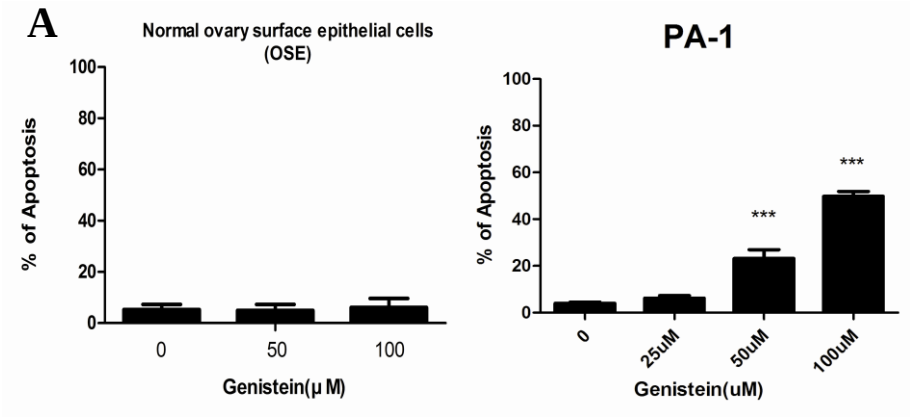
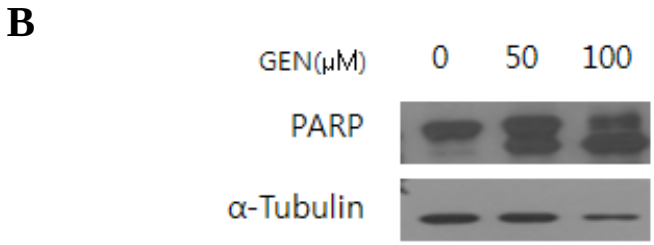

Normal ovary surface epithelial cells
(OSE)
ROS production (%)
400
300
200
100
0
0
50
100
Genistein(μ M)

Figure 2. Increased level of intracellular ROS by genistein in ovarian cancer cells

Three ovarian cancer cells were incubated with (A) 50, 100 μM of genistein for 24 hours or (B) 100 μM genistein for 6, 24 hours. (C) Normal ovarian epithelial cells (OSE) were also cultured with 50, 100 μM of genistein for 24 hours. Cells were stained with 25 μM DCF - DA for 30 min. Cellular ROS level was measured by flow cytometry. Data represent the mean ± SEM of three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. control.


A
Normal ovary surface epithelial cells (OSE)
% of Apoptosis
100
80
60
40
20
0
0
50
100
Genistein(μM)
PA-1
% of Apoptosis
***
***
0
25uM
50uM
100uM
Genistein(uM)


B
GEN(μM)
0
50
100
PARP
α-Tubulin

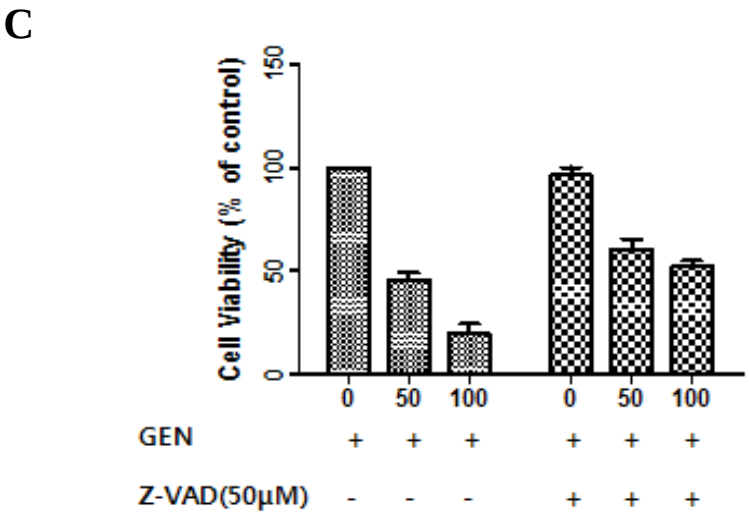

C
Cell Viability (% of control)
150
100
50
0
0
50
100
0
50
100
GEN
+
+
+
+
+
+
Z-VAD(50μM)
-
-
-
+
+
+

Figure 3. Apoptosis induced by genistein in PA-1 ovarian cancer cells

(A) The effect of genistein on the apoptosis in normal ovary surface epithelial cells and PA-1 cells. Normal ovarian surface epithelial cells and PA-1 cells were incubated with genistein (25, 50, 100 μM) for 24 hours. Apoptotic cell death was measured by flow cytometry after Annexin V & PI straining. Data represent the mean ± SEM of three independent experiments. ****P<0.001* vs. control. (B) PARP cleavage with genistein treatment in PA-1 cells. After 24 hours of treatment of genistein (50, 100 μM), cleaved of PARP was determined by Western blot. α - tubulin was used as the loading control. (C) Reversal of decreased cell viability by genistein with treatment of pan-caspase inhibitor, Z-VAD-fmk in PA-1 cells. PA-1 cells were pre-treated with or without Z-VAD-fmk (50 μM) for 1 hour before the addition of 100 μM genistein for an additional 24 hours. The cell viability of PA-1 cells was assessed by the MTT assay.

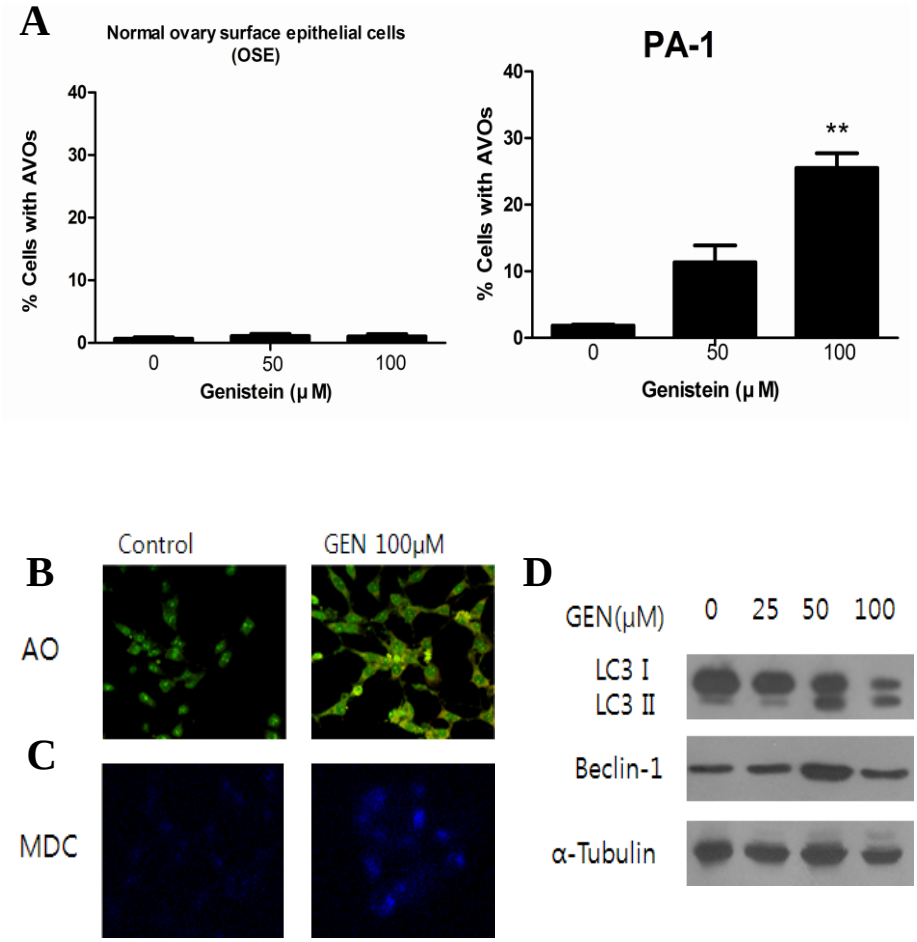

A
Normal ovary surface epithelial cells (OSE)
% Cells with AVOs
40
30
20
10
0
0
50
100
Genistein (μM)
PA-1
% Cells with AVOs
**
40
30
20
10
0
0
50
100
Genistein (μM)
B
Control
GEN 100μM
AO
C
MDC
D
GEN(μM)
0
25
50
100
LC3 I
LC3 II
Beclin-1
α-Tubulin

Figure 4. Increased autophagy by genistein only in ovarian cancer cells

(A) Autophagy by genistein in normal ovarian surface epithelial cells and ovarian cancer cells. Normal ovary surface epithelial cells and ovarian cancer cells were cultured with 50, 100 μM genistein for 24 hours. Genistein-induced autophagy was measured by flow cytometry after Acridine orange staining. Data represent the mean ± SEM of three independent experiments. ***P<0.01* vs. control (B) Increased acidic vesicle organelle (AVO) in genistein-treated ovarian cancer cells. Cells were incubated with 100 μM genistein for 24 hours and then stained with 1ug/ml acridine orange (AO) for 15 min. Cells were observed by confocal microscopy. (C) Increase of MDC stained cells in genistein-treated cells. Ovarian cancer cells were treated with genistein (100 μM) for 24 hours and incubated with 50 μM MDC for 1 hour. Cells were observed under confocal microscopy. (D) Increase in expression of autophagy-related Beclin-1 and processing of LC 3B by genistein in PA-1 cells. Cells were treated with various concentrations of genistein (0 – 100 μM) for 24 hours and analyzed using autophagy-specific markers by Western blot. α- tubulin was used as the loading control.

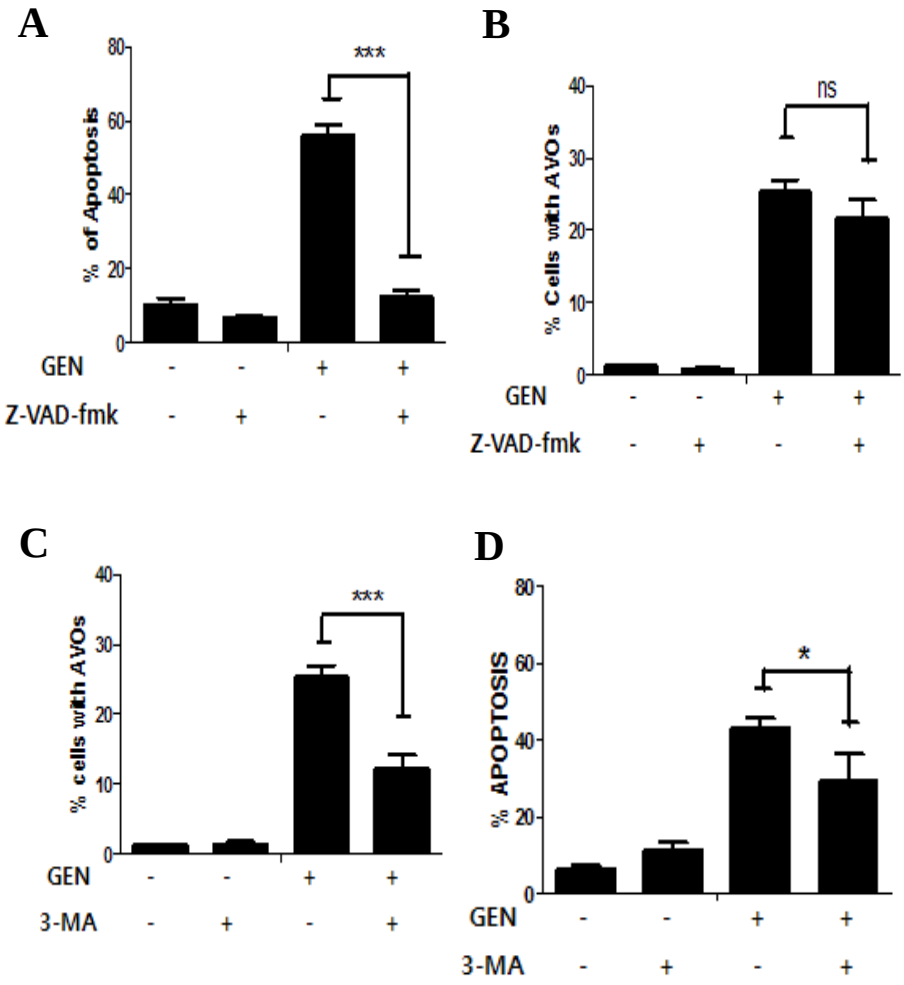

A
% of Apoptosis
80
60
40
20
0
***
GEN - - + +
Z-VAD-fmk - + - +
B
% Cells with AVOs
40
30
20
10
0
ns
GEN - - + +
Z-VAD-fmk - + - +
C
% cells with AVOs
40
30
20
10
0
***
GEN - - + +
3-MA - + - +
D
% APOPTOSIS
80
60
40
20
0
*
GEN - - + +
3-MA - + - +

Figure 5. Autophagy-mediated apoptosis by gensitein in ovarian cancer cells

(A) Cells were pre-incubated with Z-VAD (50 μM) for 1 hour before the addition of 100 μM genistein for an additional 24 hours. Annexin V/PI staining was performed to assess apoptotic cell death. Data represent the mean ± SEM of three independent experiments. ****P<0.001* GEN *vs.* GEN+Z-VAD-fmk. (B) Cells were stained with acridine orange. After staining, cells were examined by a fluorescence microscopy. (C) Cells were pre-incubated with 3-MA (5 mM) for 1 hour before the addition of 100 μM genistein for an additional 24 hours. Cells were stained with acridine orange. After staining, cells were observed by a fluorescence microscopy. Data represent the mean ± SEM of three independent experiments. ****P<0.001* GEN *vs.* GEN+3-MA. (D) Annexin V/PI staining was performed to assess apoptotic cell death. Data represent the mean ± SEM of three independent experiments. **P<0.05* GEN *vs*. GEN+3-MA.

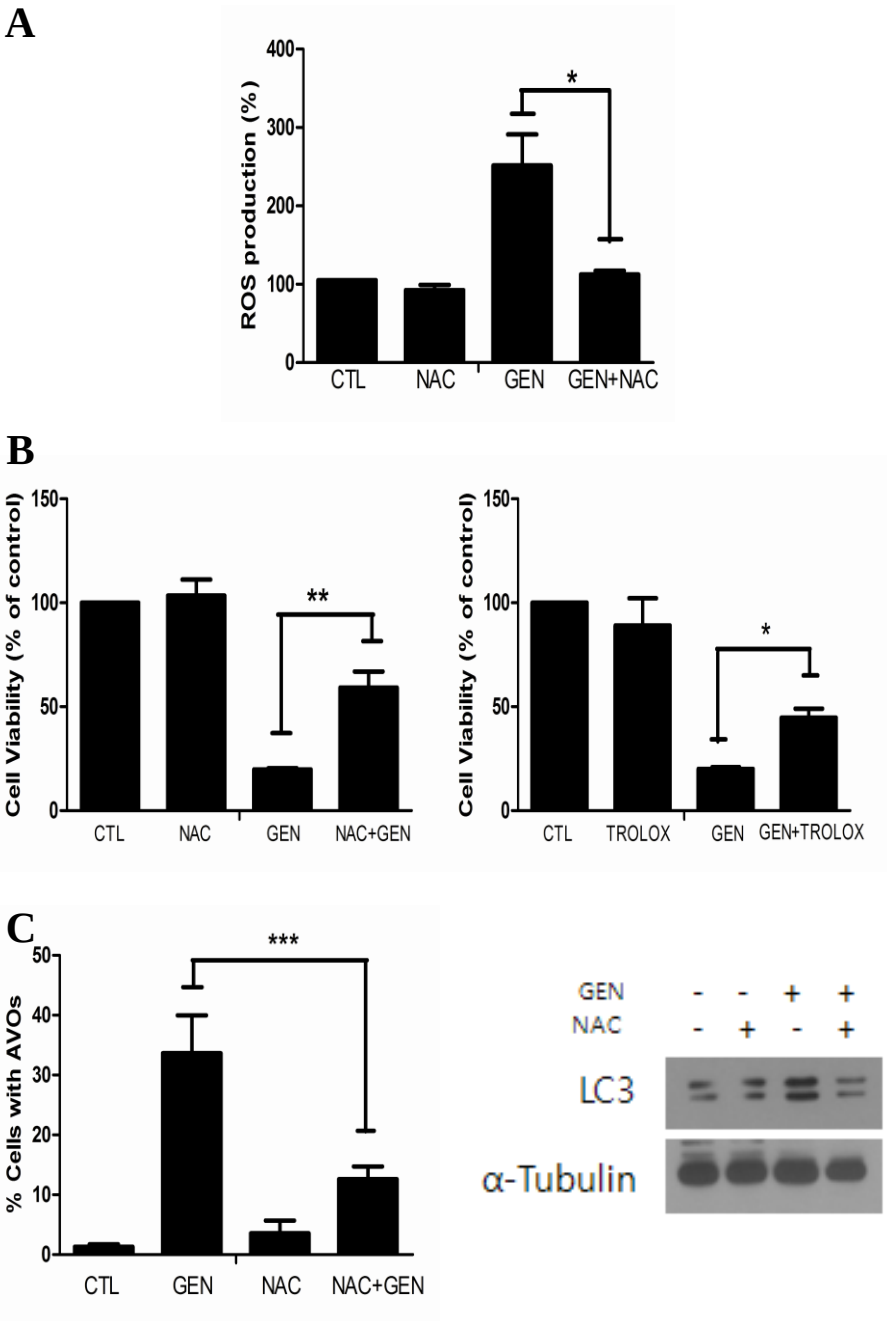

A
ROS production (%)
400
300
200
100
0
*
CTL
NAC
GEN
GEN+NAC
B
Cell Viability (% of control)
150
100
50
0
**
CTL
NAC
GEN
NAC+GEN
Cell Viability (% of control)
150
100
50
0
*
CTL
TROLOX
GEN
GEN+TROLOX
C
% Cells with AVOs
50
40
30
20
10
0
***
CTL
GEN
NAC
NAC+GEN
GEN - - + +
NAC - + - +
LC3
α-Tubulin

Figure 6. Inhibition of genistein induced autophagy by ROS scavengers

(A) PA-1 cells were pre-incubated with NAC (10m M) for 1 hour before treatment with genistein (100 μM). Intrinsic ROS levels were measured using DCF-DA by flow cytometry. Data represent the mean ± SEM of three independent experiments. ***P<0.05* GEN vs. GEN+NAC. (B) Cell growth inhibition was examined by the MTT assay. Cells were pre- incubated with NAC (10 mM) or Trolox (2 mM) for 1 hour before treated with genistein for 24 hours. Data represent the mean ± SEM of three independent experiments. ***P<0.01* GEN *vs.* GEN+NAC, **p<0.05* GEN vs. GEN+Trolox. (C) Inhibition of autophagy induction by ROS scavenger, NAC. The cells were treated with genistein (100 μM) and 10 mM NAC for 24 hours. The effect of NAC on autophagy was examined Acridine orange staining for autophagic vesicle was determined by flow cytometry and LC3 expression level by Western blot. Data represent the mean ± SEM of three independent experiments. ****P<0.001* GEN vs. GEN+NAC.

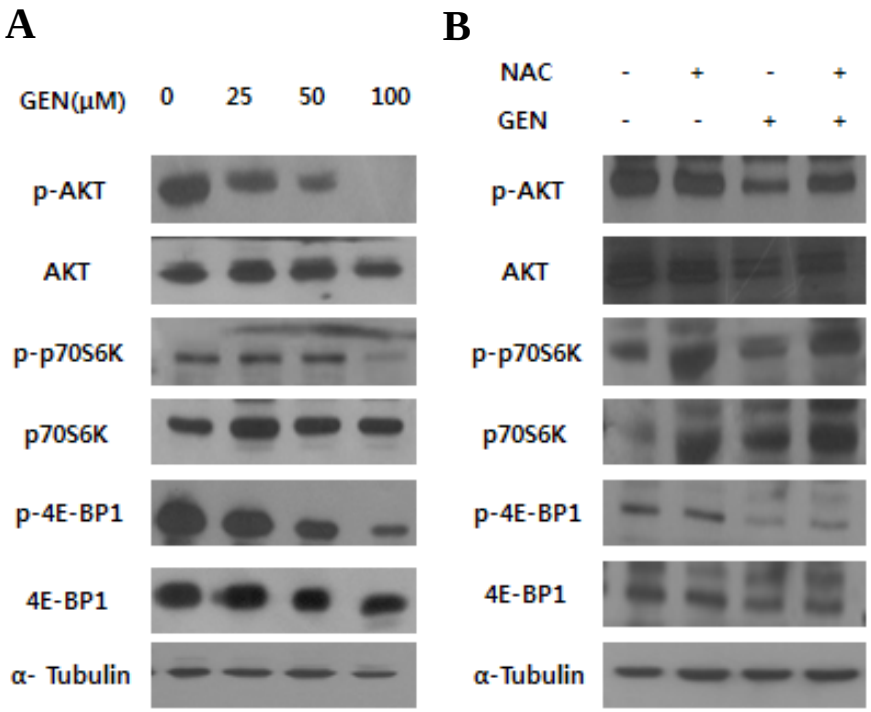
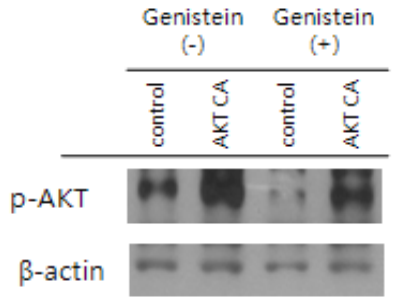

A
GEN(μM)
0
25
50
100
p-AKT
AKT
p-p70S6K
p70S6K
p-4E-BP1
4E-BP1
α- Tubulin
B
NAC
GEN
p-AKT
AKT
p-p70S6K
p70S6K
p-4E-BP1
4E-BP1
α-Tubulin

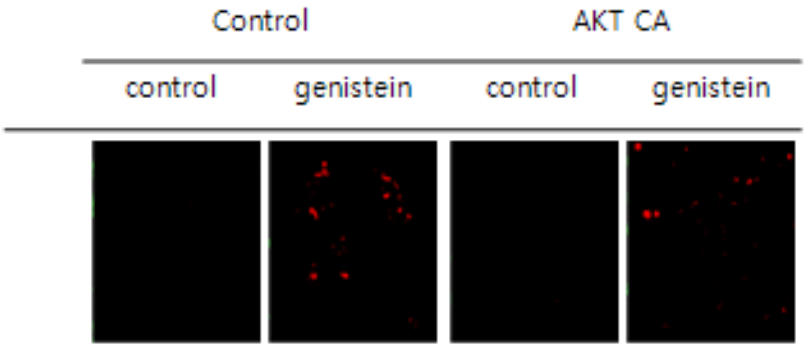
Figure 7. Inhibition of AKT/mTOR signaling by genistein-mediated ROS generation

(A) After 24 hours of treatment with 25, 50 or 100 μM genistein (GEN), Akt/mTOR signaling molecules were detected by Western blot. (B). After 24 hours of treatment with 100 μM genistein and/or 10 mM NAC in PA-1 cells, the expression levels of Akt/mTOR signaling molecules were subjected to Western blot.

A


Genistein (-)
Genistein (+)
control
AKT CA
control
AKT CA
p-AKT
β-actin

B


Control
AKT CA
control
genistein
control
genistein

C

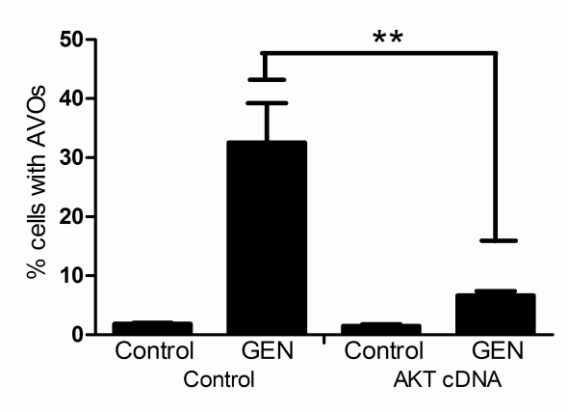

**
% cells with AVOs
50
40
30
20
10
0
Control
GEN
Control
GEN
Control
AKT cDNA

Figure 8. Inhibition of genistein-induced autophagy by overexpression with a constitutively activated Akt (CA-Akt)

PA-1 cells were transfected with active Akt cDNA and then treated with genistein (100 μM) for 24 hours. (A) Upregulation of Akt phosphorylation by active Akt cDNA transfection was verified by Western blot. Induction of autophagy in CA-Akt transfected cells treatment with gensitein. CA-Akt transfected cells were incubated with 100 μM genistein for 24 hours. (B) The acridine orange staining was performed to assess autophagy induction by confocal microscopy. (C) The percentage of autophagic vesicle organelle was assessed by acridine orange stain. Data represent the mean ± SEM of three independent experiments. ***P<0.01* GEN vs. CA-Akt + GEN

DISCUSSION

In this study, we showed for the first time that, genistein-induced ROS generation caused autophagic cell death in ovarian cancer cells. Also an autophagy inhibitor, 3-MA, effectively blocked ROS-mediated cell death by genistein. Furthermore, ROS scavenger also effectively inhibited autophagic cell death. In contrast, there was no effect on normal ovarian surface epithelial cells in relation to the ROS generation and autophagic cell death.

The targeted therapy is a crucial issue in cancer treatment. Recently, many studies have focused on the difference of ROS level between cancer and normal cells. Cancer cells could produce higher levels of ROS than normal cells. Raj et al. (38) reported that ROS generation by piperlongumine selectively induced the death of cancer cells indicating that enhancing ROS generation in cancer cells could be a strategy for a cancer-selective therapy. The pro-oxidant action of natural compounds may be a crucial mechanism for their anticancer properties (39). Genistein can induce the generation of ROS in a variety of cancer cells (19). Jiang et al. (40) reported that genistein synergized with arsenic trioxide (ATO) to significantly increase the generation of ROS and reduce cytotoxicity in human hepatocellular carcinoma (HCC). In line with previous studies, our results showed that genistein could significantly increase intrinsic ROS level in ovarian cancer cells, in contrast to normal ovarian epithelial cells. Therefore, our results implicate that genistein can enhance ROS generation and that this pro-oxidant property could be

possibly be used in targeting anticancer effects.

Autophagy is involved in cellular homeostasis in normal cells. But, the role of autophagy in cancer is controversial. Depending on the environmental condition, it can induce either cell survival or cell death. Recent studies showed that autophagic cell death, known as type II programmed cell death, is response to anticancer therapies in various cancer cells. We demonstrated that genistein induced autophagy in ovarian cancer cells and that genistein-induced autophagy is leading to cell death. Consistent with our study, Hung's (41) group found that 6-shogaol, an active constituent of dietary ginger, induces autophagic cell death in human non-small cell lung cancer A549 cells. Y Wang et al. (29) reported that piperlongumine induces autophagic cell death in human osteosarcoma cells through activation of p38 signaling. Several studies showed that ROS could mediate apoptosis and autophagy (8, 42). Chen et al. (8) showed that oxidative stress, induced by H2O2 or 2-methoxyestradiol (2-ME), causes autophagic cell death in HEK 293, U87, and HeLa cells. Also, Kim's group demonstrated that a ginseng metabolite, compound K, induced both autophagy and apoptosis through ROS generation and JNK activation in human colon cancer cells (43). Consistent with previous studies, we demonstrated that genistein induced autophagy and inhibited cell growth via ROS generation. Application of NAC or Trolox, ROS scavengers, significantly reduced genistein-induced autophagy and cell death. Therefore, our results support the notion that under oxidative stress, ovarian cancer cells can be killed by autophagy.

The Akt/mTOR signaling pathway is the major negative regulator of

autophagy and cell death. Recent reports suggested that the ability of the Akt/mTOR pathway to induce autophagy in response to ROS has a role in cell death. For example, Poornima et al. (44) recently reported that neferine-induced autophagy is mediated through ROS generation and subsequent inhibition of Akt/mTOR . In this study, we found that the constitutive active form of Akt suppressed the genistein-induced autophagy, suggesting that Akt inhibition is necessary for the action of genistein. In addition, we demonstrated that ROS generation by genistein resulted in the inhibition of Akt/mTOR signaling pathway in ovarian cancer cells. This result is consistent with the report by Shin et al. (45) that omega-3 polyunsaturated fatty acid DHA induces autophagy via ROS-mediated Akt/mTOR inhibition in prostate cancer. Thus, all together, our results suggest that Akt/mTOR signaling is abolished though the oxidative stress by to genistein, causing autophagic cell death.

Taken together, the results presented here provide an insight into the role of ROS in death of ovarian cancer cells by genistein. ROS generation could play an important role in genistein-induced autophagic cell death through the inhibition of Akt/mTOR signaling pathway in ovarian cancer cells. This may lead to new strategies to selectively target cancer cells using ROS and an autophagy inducer in ovarian cancer.

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국문 초록

암 세포는 산화 환원 반응의 불균형으로 인해 활성 산소에 취약하다. 최근 폴리 페놀릭 물질 중 하나인 genistein이 유방암, 전립선암, 혈액암 등의 다양한 타입의 종양에서 산화촉진제로 작용하여 세포 사멸을 유발하는 것이 연구되고 있다. 본 연구에서, 우리는 난소암 세포주에서 genisteim에 의해 유발된 자가포식과 활성화 산소의 발생을 통한 세포 사멸을 연구 하였다. Genistein에 의해 유도되는 활성 산소의 발생은 세포 자살과 자가 포식을 유발한다. 또한 자가 포식 억제제인 3-MA를 전처리 하였을 경우, 난소암 세포주의 genistein에 의한 세포 사멸이 억제됨을 보였다. 이러한 결과들은 자가 포식 과정이 genistein에 의해 유발되는 세포 사멸에 관여 함을 제안한다. 흥미롭게도, genistein에 의해 유발되는 세포 사멸은 활성 산소 저해제, NAC, Trolox 등의 물질에 의해 회복됨을 보였고, 또한 NAC 처리시, genistein에 의해 유발되는 자가 포식 과정 또한 감소되는 것을 보아, 우리는 genistein에 의한 자가 포식 세포 사멸에 활성 산소의 발생이 중요한 역할을 하는 것을 제안 할 수 있다. 게다가, genistein에 의해 저해되는 AKT/mTOR 신호 전달 과정에도 활성산소가 관여하는 것을 알 수 있다. 종합해 보면, 본 연구에서는 AKT/mTOR 신호 전달 저해와 활성산소에 의한 자가포식의 유발을 통한 genistein의 세포 사멸과정이 암세포

사멸에 중요한 역할을 함을 제안한다.

주요어: 활성 산소(ROS), 자가포식(Autophagy), Genistein, 난소암 (ovarian cancer), 세포 자살(apoptosis)

학 번: 2010-23728