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황금의 유방암세포주에 대한 항암작용

용형순[a], 고성규*, [a,b]

상지대학교 한의과대학 내과학교실[a], 서울대학교 의과대학 암연구소 종양생물학교실[b]

Inhibition of cellular proliferation and apoptosis by *Scutellaria Baicalensis* in MDA-MB-231 breast cancer cells

Hyung-Soon Yong[a], Seong-Gyu Ko *[a,b]

Department of Internal Medicine, College of Oriental Medicine, SangJi Universitya
Department of Tumor Biology, Cancer Research Institute, College of Medicine, Seoul National Universityb

Objective : 황금의 유방암세포주에 대한 항암효과 및 기전에 대한 연구는 아직 미흡하며, 특히 에스트로젠리셉터를 가지지않은 유방암세포주인 MDA-MB-231에 대한 효과 및 기전에 대한 연구는 아직 발표된바 없어, 이에 대한 연구가 진행되었다.

Methods : 인간 유방암세포주 MDA-MB-231 MTT assay를 이용 성장방해비율을 조사하였으며. FACS analysis를 이용 cell cycle analysis를 시행하였고, Western Blot Analysis 및 Annexin Ⅴ analysis를 시행하였다.

Results : MDA-MB-231에 대한 황금의 IC50는 180 ug/ml 이었으며 최대 세포성장억제효과는 500㎍/ml로 한약재중 비교적 강한 세포독성을 보여주었다. 유세포분석에서 황금 500㎍/ml의 농도를 72시간 투여한 경우 세포사멸(Sub G1) 분율이 대조군의 1.7%에 비해 21%로 높아 현저한 용량의존적인 세포사멸현상을 보여주었으며, 세포사멸을 보다 명확히 규명할 수 있는 Annexin Ⅴ analysis에서도 황금 200㎍/ml농도일때 48시간에서 17%의 뚜렷한 세포사멸효과를 나타내었다. 한편 세포사멸촉진인자인 Bax, 세포사멸실행단백질인자인 caspase 3의 활성과 PARP의 분할은 세포사멸이 세포주기정지와 더불어 세포사멸의 과정에 p53이 관여함을 알 수 있다. 앞으로의 연구는 p53발현이 다른 세포주와 각 단백질의 억제제를 통해 인과적인 관련성을 좀 더 명확히 할 필요가 있어야 할 것으로 생각되어진다.

Conclusion : 유방암의 예후에 있어 호르몬치료에 부적절함으로 인해 예후가 나쁜 에스트로젠리셉터 발현이 없는 유방암에 대해서도 황금이 탁월한 항암효과를 보여주고 있으며, 임상적으로 황금단독, 다른 항암약재와의 배합, 그리고 기존의 항암화학요법이나 방사선요법과의 병용투여를 통한 초기 및 진행된 유방암의 치료에 대한 새로운 접근의 실마리를 제공할 것으로 생각된다.

주요어 : 황금, 세포자연사, 유방암

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· Corresponding Author : Seong-Gyu Ko, O.M.D., Ph.D.
Department of Internal Medicine, College of Oriental Medicine, Sangji University 283 Woosan-dong, Wonju-si, Kangwon-do, Korea, 220-130
(Phone : 033-741-9209, FAX : 033-732-2124, E-mail : sgko9209@snu.ac.kr)
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INTRODUCTION

The dried root of *Scutellaria baicalensis* George, also known as Huang Qin, has been widely used as a traditional medicine in Korea, China, and Japan. This medicinal herb has been used for anticancer, antiviral,

antibacterial, and anti-inflammatory purposes[1,2,3,4,5,6]. Recent studies have also shown that *Scutellaria baicalensis*, alone or in combination with other herbs, can inhibit cancer cell growth or induce apoptosis in breast, hepatocellular, pancreatic, prostatic, and urothelial carcinoma cell lines[7,8,9,10,11].

Despite improvements in treatment strategies against various cancer types during the past 40 years, breast cancer still remains as one of the main cause of cancer mortality among women in the whole world. Therefore, it is important to mechanistically examine if natural products such as *Scutellaria baicalensis* extract may have chemo-preventive and therapeutic activities against breast cancers. Recently it was reported that *Scutellaria baicalensis* extract has anticancer activity on head and neck squamous cell carcinoma (HNSCC) *in vitro* and *in vivo*, and that the anticancer activity attributes to inhibition of prostaglandin E2 (PGE2) synthesis via suppression of cyclooxygenase-2 (COX-2) expression. In addition, the effects appear to be via synergisticeffects among active components in *Scutellaria baicalensis* extracts[6].

However, the mechanistic aspects how *Scutellaria baicalensis* extract may affect breast cancers are much unknown. The aim of this study is to understand how *Scutellaria baicalensis* extract affects the viability of MDA-MB-231 breast cancer cells, especially with regards to intracellular molecules related to cell cycle progression and apoptosis.

MATERIALS AND METHODS

Preparation of medicinal herbs

The raw extract of *Scutellaria baicalensis* was purchased from Dongwoodang Pharmaceutical Co. (Seoul, Korea) and prepared by sonication of dried root of the plant in ethanol 80% and then the ethanol extract underwent a freeze-drying step. The powder form of the extract was dissolved in RPMI 1640 medium (Life Technologies, Inc., Rockville, MD) to 10 mg/ml, vortexed at room temperature for 1 min, and incubated at 37℃ for 1 hr while rotating. Then this solution was centrifuged at 12,000 rpm for 5 min to remove any insoluble ingredients. The supernatant was then passed through a 0.22-m filter for sterilization and diluted with RPMI 1640 culture medium to final concentrations of 6.25 ~1,000 g/ml, before use.

Cell Lines and Tissue Culture

A human breast cancer cell line, MDA-MB-231, was purchased from the ATCC and grown in RPMI 1640 containing 10% FBS (Hyclone Laboratories, Inc., Logan, UT) and 1% gentamicin in a 5% CO2 humidified atmosphere. Subconfluent monolayers of cells were used in all experiments.

Growth Inhibition Assay

To investigate of the effect of *Scutellaria baicalensis* on MDA-MB-231 cell growth, cell viability was determined by measuring MTT dye absorbance of viable cells at various times after the extract treatment. Ten thousand cells per well was seeded onto a 96-well microtiter plate (Nunc, Roskilde, Denmark). Twenty four hours later, cells were treated with various concentrations of *Scutellaria baicalensis* extract, and incubated for an additional 3 days at 37℃. Subsequently, 50 l of MTT (Sigma) at a concentration of 2 mg/ml was added to each well, and cells were then incubated for an additional 4 h at 37℃. The supernatant was aspirated, and 150 l of DMSO were added to the wells. The absorbance was then measured at a wavelength of 570 nm using an ELX800 reader (Bio-Tek Instruments, Inc., Winooski, VT). The IC50 was determined, assuming the viability of untreated cells to be 100%, expecting that cell death in control would be negligible.

DNA contents Analysis

A total of 5 x 105 cells were seeded in 60mm dishes and incubated for 24 h at 37℃. *Scutellaria baicalensis* extract at the various concentrations indicated were directly added to the dishes and incubated for additional 24, 48 or 72hrs. Cells were then washed twice with ice-cold PBS, and then supernatant (containing spontaneously detached cells) and adherent cells were collected, fixed by addition of 4 ml 70% ethanol, and stored at -20℃ for at least 30 min. Cells were then spun down, washed twice with ice-cold PBS, incubated in PBS containing 10 mg/ml of RNase A (Sigma) for 15 min at 37℃, and stained with 10 mg/ml of propidium iodide (PI). The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA, by using a FACStar flow cytometer (Becton Dickinson, San Jose, CA).

Annexin V staining

Annexin V staining was performed to detect the externalized phosphatidylserine during the apoptosis progression, by using an annexin V assay kit (Pharmingen, San Diego, CA, USA) according to the manufacturer' s recommendations. In brief, trypsinized cells were washed twice with ice-cold PBS and then resuspended in a binding buffer (10 mM HEP-ES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Then, both 5 ml of annexin V-FITC (Pharmingen, San Diego, CA) and 10 ml of 20 mg/ml PI were added to the cells, which were later analyzed with a FACSCalibur flow cytometry (B&D, Mountain View, CA).

Western blot and co-immunoprecipitation analysis

MDA-MB-231 cells were seeded in 100 mm culture dishes, treated with or without the *Scutellaria baicalensis* extract, and incubated for the indicated periods. After incubation, lysates were prepared from the cells using a lysis buffer containing 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM phenylmethy-lsulfonyl fluoride, 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 g/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, and 1 mM benzamidine, and then incubated on ice for 30min. Lysates were then cleared by centrifugation at 13,000 rpm at 4℃ for 20 min. For a co-immunop-recipitation of Cdk2 with cyclin E, lysates with the same protein amount were mixed with rabbit polyclonal anti-Cdk2 antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA), rolled over at 4℃ for 4 hr, mixed further with protein A/G beads, and rolled over again at 4℃ for another 2 h. After the incubation, SDS-PAGE sample buffer were added to the immunopereciptates before a boiling. Twenty mg of proteins or immunoprecipitates were fractionated by SDS-polyacrylamide electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in a blocking buffer containing 5% skim milk, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 and then probed with various primary antibodies, following by a chemiluminescence detection (ECL, Amersham Pharmacia Biotech) with autoradiography.

Statistical Analysis

Results shown are representative of at least three independent experiments performed in triplicate and are presented as the mean standard deviation (SD).

RESULTS

Growth inhibition of MDA-MB-231 breast cancer cells by *Scutellaria Baicalensis* The growth inhibition of MDA-MB-231 by the *Scutellaria Baicalensis* extract

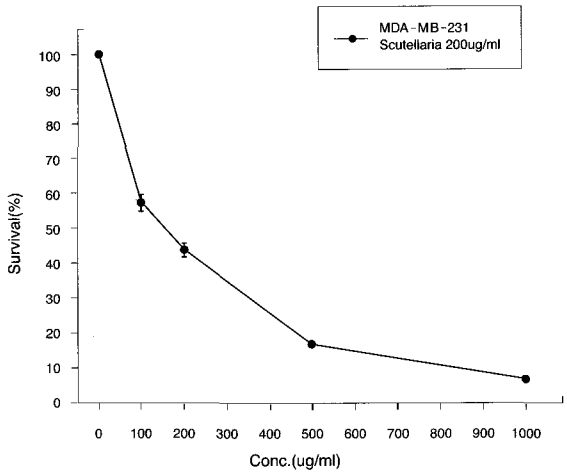

Fig. 1. Growth inhibition of MDA-MB-231 breast cancer cells by *Scutellaria baicalensis*extract treatment. MDA-MB-231 cells were seeded to wells of 96 well plates in the presence of normal culture media at 1.0 x 104 cells/well. Twenty four hours later, cells were treated with indicated concentrations of *Scutellaria baicalensis*extract for additional 72 hrs. Then MTT assay was performed as explained in Material and methods. Each condition was in triplicate. Data were shown as mean standard deviation (SD) and are representative from three independent experiments.

was determined by quantifying viable cells in the absence or presence of treatment of *Scutellaria Baicalensis* extract at various concentrations for 3 days. As shown in Fig. 1, *Scutellaria baicalensis* extract significantly decreased cell viability in a dose-dependent manner. Based on the growth inhibition curve, the concentration of *Scutellaria Baicalensis* extract required for 50% inhibition of growth (IC50) for MDA-MB-231 cells was about 180mg/ml. The maximal inhibition of cell growth (>80%) was achieved at 500 g/ml in MDA-MB-231cells. A time-dependent growth inhibition of MDA-MB-231 cells was also observed with a maximal growth inhibition within 2 days after the treatment (data not shown).

Apoptosis by *Scutellaria Baicalensis* extract

In order to examine the mechanism of growth inhibition by the *Scutellaria baicalensis* extract, MDA-MB-231 cells treated with *Scutellaria baicalensis* extract at various concentrations from 100 to 500mg/ml for 72 hrs were analyzed for DNA contents by flow cytometric analysis. Flow cytometric results showed that treatment of *Scutellaria baicalensis* extract caused significant apoptosis in a dose-dependent manner; treatment of 500 mg/ml *Scutellaria Baicalensis* extract resulted in 21% apoptotic population, compared to the control of 1.7% rate (Fig. 2A). Furthermore, when apoptotic rates by treatment of 200 mg/ml *Scutellaria Baicalensis* extract for various periods were examined, the rates were in a time-dependent manner (Fig. 2B). In addition, the apoptotic population after treatment of *Scutellaria Baicalensis* extract was also quantified by FACS analysis of annexin V stained cells, in which the apoptotic rate was shown as about 17% by treatment of

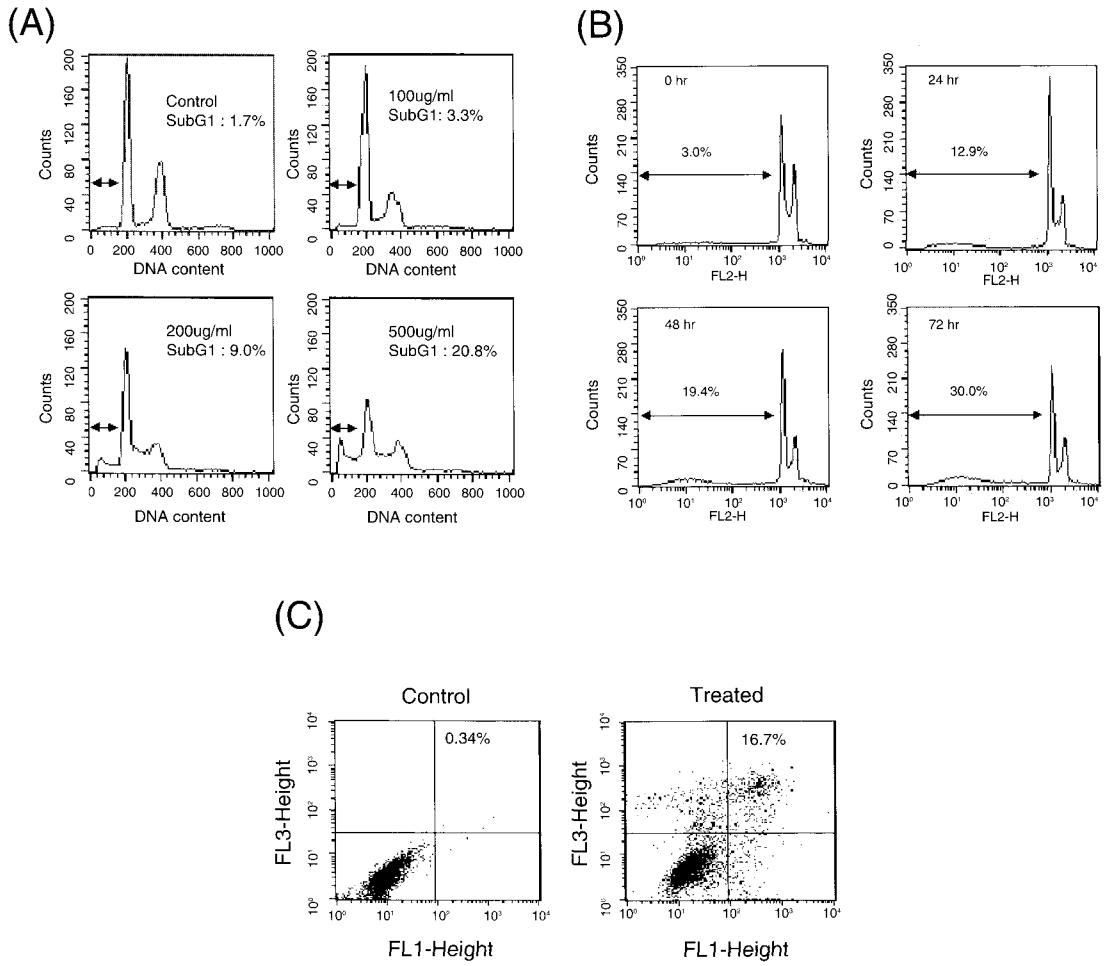

Fig. 2. Apoptosis of MDA-MB-231 cells by *Scutellaria baicalensis* extract treatment. Cells in 60 mm culture dishes were treated with 200 mg/ml *Scutellaria baicalensis* extracts for indicated periods. The treatment was done by a direct addition of *Scutellaria baicalensis* extract solution into the culture media.After incubation, cells floating and adherent were collected and combined before PI staining and flow cytometric analysis for cells with subG1 DNA contents, as explained in the Materials and methods. Shown data are representative from three independent experiments. (A) A dose-dependent apoptosis by treatment of *Scutellaria baicalensis* extract. (B) A time-dependent apoptosis by *Scutellaria baicalensis* extract treatment at 200 mg/ml. (C) Quantification of apoptosis by *Scutellaria baicalensis* treatment via annexin V staining. Cells were untreated (left histogram) or treated (right histogram) with 200 mg/ml *Scutellaria baicalensis* extract for 48 h, prior to flow cytometric quantification of cells stained with annexin V, as explained previously (Lee and Juliano, 2000). Data shown was representative from two independent analyses.

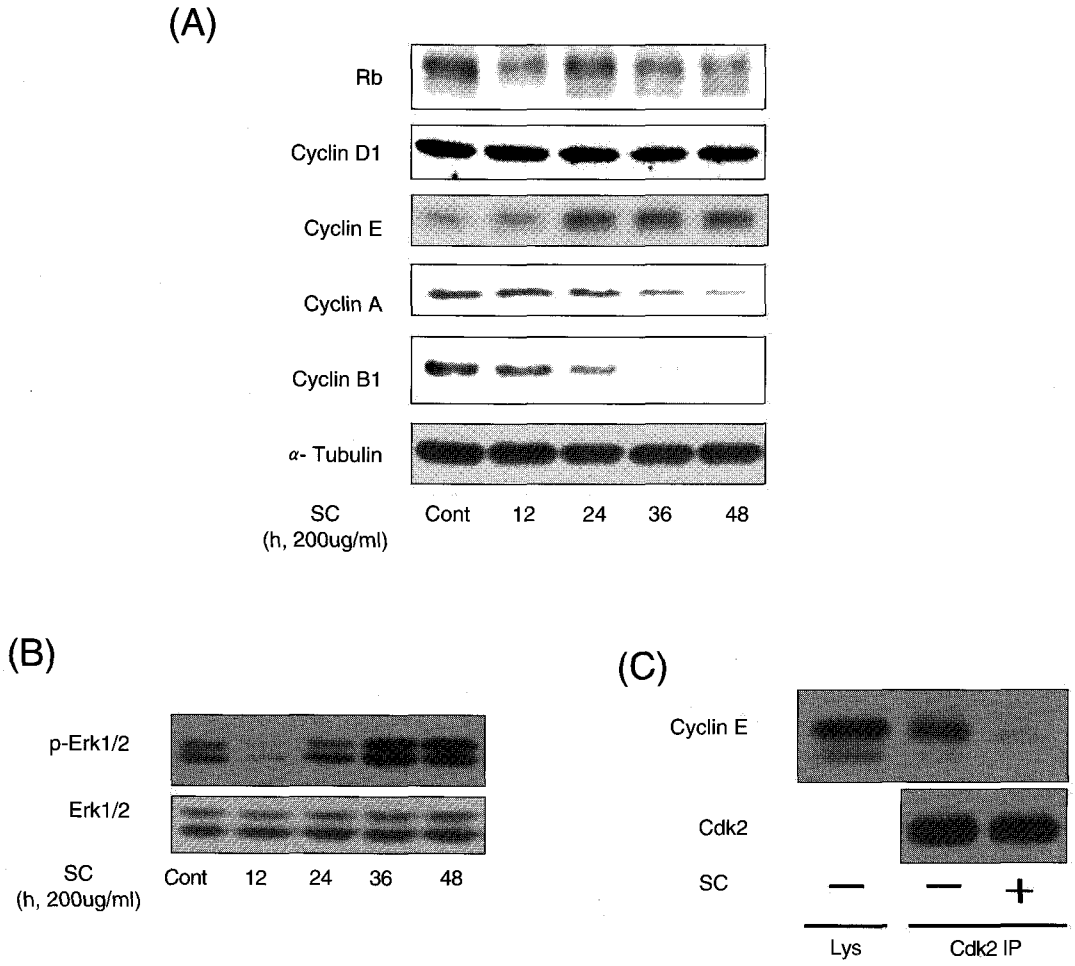

Fig. 3. Regulation of cyclins expression, Erk1/2 activity, and complex formation between Cdk2 and cyclin E by *Scutellaria baicalensis* extract treatment. Cells in 100 mm culture dishes were treated with 200 mg/ml *Scutellaria baicalensis*extract for indicated periods. After treatment, cells were washed twice with ice-cold PBS and then lysates were prepared using a RIPA lysis buffer. Lysates normalized to have equal protein amounts were used for immunoblots by SDS-PAGE using primary antibodies against the indicated molecules, as described in the Materials and methods. The data are representative of at least three isolated experiments.(A and B) Lysates prepared as explained above were used for immunoblottings using the antibodies against the indicated molecules. (C) Lysates were mixed with rabbit polyclonal anti-Cdk2 antibody and an immunoprecipitation was performed. Then the immunoprecipitates were analyzed by an immunoblot using mouse anti-cyclin E antibody to examine the complex formation of cdk2 with cyclin E, or anti-cdk2 antibody for an equal immunoprecipitation of cdk2 from each condition.

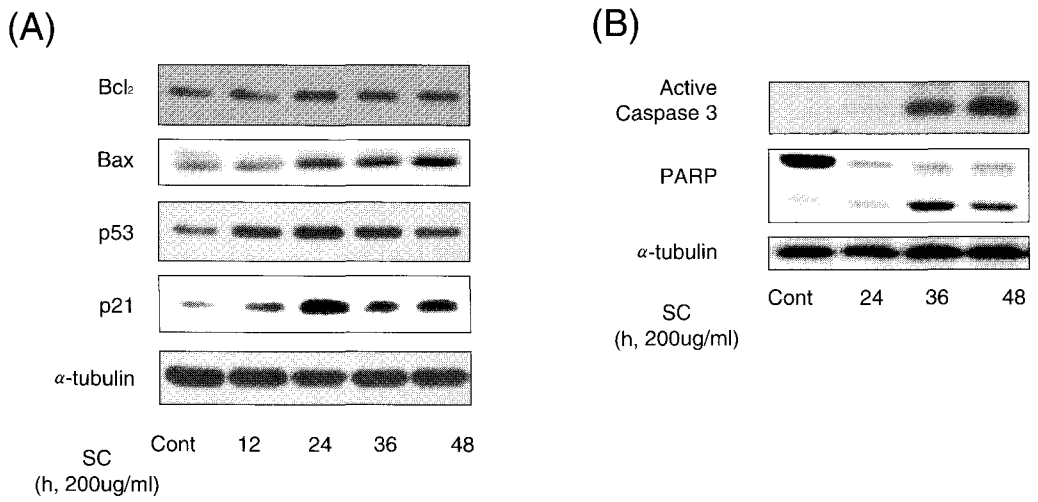

Fig. 4. Increases in and activation of pro-apoptotic molecules by *Scutellaria baicalensis*extract treatment. (A and B) Cells in 100 mm culture dishes were treated with 200 mg/ml *Scutellaria baicalensis*extract for indicated periods. After treatment, lysates were prepared using a RIPA lysis buffer, as explained above. Lysates were used for immunoblots by SDS-PAGE using primary antibodies against the indicated molecules. The data are representative of at least three isolated experiments.

200 mg/ml *Scutellaria Baicalensis* extract for 48 h (Fig. 2C). Therefore, these observations indicate that the growth inhibitory effect by *Scutellaria Baicalensis* extract involved apoptosis.

Western Blot Analysis

While the apoptotic analysis by determination of cells with subG1 DNA contents (Fig. 2A), G1-growth arrest was shown. Therefore, it is likely that the growth inhibition by *Scutellaria Baicalensis* extract might involve apoptosis and G1-growth arrest as well. Therefore, we have examined expression levels of cell cycle-related molecules, especially cyclins. A shown in the Fig. 3A, G1 phase cyclins including D1 and E were highly maintained even under the conditions that apoptotic populations were obviously observed. Furthermore when phosphorylation of retinoblastoma protein (pRb) was examined biochemically, it was shown that pRb became hypophosphorylated over time of the treatment, indicating that the *Scutellaria Baicalensis* extract-treated cells might not be efficient to traverse the restriction ('R') point in the G1 cell cycle phase (Fig. 3A). Being consistent with this idea, cyclins A and B1, S and G2/M phase cyclins, respectively, were reduced as time passed after the treatment (Fig. 3A). These again indicate that entry into S phase was not allowed by the treatment. Therefore, it is likely that treatment of *Scutellaria Baicalensis* extract might cause

apoptosis via growth arrest at G1 phase.

Next we had interests in understanding of how expression of cyclins D1 and E were highly maintained and how cyclin A was reduced by the *Scutellaria Baicalensis* extract treatment. Therefore, we examined the activities of Erk1/2, which is a major upstream molecule to induce cyclin D1 upon growth factor-mediated mitogenic signaling. It was highly maintained even at 48 h after the treatment, being consistent with the highly maintained level of cyclin D1 under the same condition (Fig. 3B). Generally the expression of cyclin E is a prerequisite of an induction of cyclin A. The complex between Cdk2 and cyclin E phosphorylates pRb, leading to release of E2F transcription factor to induce cyclin A (Obaya and Sedivy, 2002). Therefore, we immunoprecipitated Cdk2 in lysates untreated or treated with the *Scutellaria Baicalensis* extract for48 hr, and the immunoprecipitates were immunoblotted with anti-cyclin E antibody to examine the complex formation of Cdk2 with cyclin E. As shownin Fig. 3C, the complex formation was not significant in the condition treated with the *Scutellaria Baicalensis* extract. Therefore, although Erk1/2 activity was highly maintained to induce cyclin D1 and thereby cyclin E, cyclins A was not highly maintained. This may be the case because no significant complex formation of Cdk2 with cyclin E was available, thereby leading to hypophosphorylation of pRb which constrains E2F transcription factor for cyclin A expression.

Next we have confirmed intracellular apoptotic events biochemically, by examining the expression levels of pro-apoptotic molecules such as PARP, active caspase 3, and Bax, and anti-apoptotic molecules including Bcl2. Cell lysates were prepared at various time points after treatment of *Scutellaria Baicalensis* extract and used for Western blots. As shown in the Fig. 4A, the level of anti-apoptotic Bcl2was not altered significantly by the extract treatment, but expression of pro-apoptotic Bax increased gradually with the treatment. It was also obviously shown that induction of p53 and its downstream targets including Bax and p21, indicating that the apoptotic effectsof the extract might involve p53 action to induce Bax (leading to apoptosis) and p21 (leading to G1 arrest) (Fig. 4A). Furthermore, levels of active caspase 3 and cleaved PARP clearly increased, as time passed after the extract treatment (Fig. 4B). This implies that caspase activation and thereby cleavage of functional proteins occurred by the treatment, leading to cell death.

DISCUSSION

In this study, treatment of *Scutellaria Baicalensis* extract has shown a cytostatic activity in MDA-MB-231 breast cancer cellsvia G1 arrest not allowing S phase entry and apoptosis. The cytostatic effects also involved increased expression and activation of pro-apoptotic molecules.

Scutellaria baicalensis extract has shown a broad spectrum of biological activities, including anti-inflammatory and anticancer activity, and been used in clinical applications for a long time. This study confirmed that *Scutellaria baicalensis*has a dose-dependent anti-proliferative activity in MDA-MB-231 cells, with an IC50 of 180mg/ml (Fig. 1). Interestingly, the treatment did show increased levels of cyclins D1 and E, being supported via highly activated Erk1/2 by the treatment. On the other hand, cyclins A and B1 responsible for S and G2/M cell cycle phases were reduced significantly by the treatment, indicating that no S phase entry was allowed upon the treatment. The proportional co-relationship between cyclins E and A was disrupted due to no efficient complex formation between cyclin E and Cdk2 and thus hypophosphorylation of Rb protein upon the treatment. This G1 arrest was also well-correlated with the induced levels

of p53 and its downstream target of p21 (Fig. 4A), both of which known to arrest cells at the G1 phase[12].

The effects of *Scutellaria Baicalensis* extract treatment also resulted in an increased apoptosis via increased levels of pro-apoptotic molecules such as Bax, active caspase 3, and PARP, but not significant alteration of anti-apoptotic Bcl2. This indicates that MDA-MB-231 cells treated with *Scutellaria Baicalensis* extractmight undergo the intracellular apoptotic events, such as caspase activation, cleavages of functional proteins, and DNA breakage, in addition to G1-arrest. Furthermore, an increased expression of Bax might involve p53-mediated expression regulation. Consistently, treatment of *Scutellaria Baicalensis*extract resulted in an increase of p53 expression (Fig. 4B). Therefore, the increased level of p53 might have a dual function; it induces Bax (for apoptosis) and p21 (for G1-arrest) as well. Although p53-mediated induction of p21 might arrest cell also at G2 phase13, it was not shown in this system.

It is very likely that the *Scutellaria baicalensis* extract exerts its anticancer activities by multiple mechanisms. The extracts contain four major flavonoids, such as baicalin (80%), wogonoside (16%), baicalein (2%), and wogonin (1%), in addition to other compounds in trace amounts.14,15 Recently it was reported that an active component of *Scutellaria baicalensis* extract, baicalein, showed no anti-proliferative activity, whereas *Scutellaria baicalensis* extractsshowed a strong anti-proliferative activity to head and neck squamous cell carcinoma (HNSCC), indicating that active components of the *Scutellaria baicalensis* extract might have synergistic effects on the cells[6]. Because of a poor water solubility of pure flavonoids, such as baicalein (data not shown), use of the herb extract is more attractive, probably due to its excellent water solubility and bioavailability. Furthermore, this study may give a mechanistic insight to research fields to develop and search for more effective treatment reagents using herbal medicines, with regards to prevention of breast carcinogenesis.

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