Cent. Eur. J. Med. • 9(2) • 2014 • 193-199
DOI: 10.2478/s11536-013-0269-z

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Central European Journal of **Medicine**

Scutellarein ameliorates tongue cancer cells via mitochondria

Research Article

Guangping Jing[1,#], Jinhua Zheng*[2], Xueyong Wang[2], Haixia Li[2], Xiaohui Jiao[3]*

1 Department of Oral Anatomy & Physiology, Stomatological College, Harbin Medical University, Harbin 150001, China

2 Department of Anatomy, Basic Medical Science College, Harbin Medical University, Harbin 150001, China

3 Department of Oral & Maxillofacial Surgery, Stomatological College, Harbin Medical University, Harbin 150001, China

 # These authors contributed equally

Received 7 May 2013; Accepted 19 September 2013

Abstract: To investigate the mechanism in Scutellarein-induced apoptosis of SAS human tongue cancer cells, inhibitory effects of Scutellarein on SAS cells were detected by MTT assay. Cell apoptosis was analyzed by flow cytometry. Ultrastructural changes of SAS cells were observed by transmission electron microscopy (TEM). Mitochondrial transmembrane potential ($\Delta\Psi m$) were analyzed by JC-1 [5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide]. Western blotting was used to examine the expression level of Bcl-2, Bax and caspase-3 in SAS cells treated with Scutellarein. Scutellarein inhibited the proliferation of SAS cells in a time and dose-dependent manner and increased the percent of apoptotic cells. The mitochondrial cristae were swollen and had vacuolar degeneration. $\Delta\Psi m$ decreased when the concentration of scutellarein increased. Scutellarein effectively up-regulated the expression of mitochondrial Bax and caspase-3 and down-regulated the expression of Bcl-2. Scutellarein induces apoptosis of SAS human tongue cancer cells via activating mitochondrial signaling pathway.

Keywords: *Scutellarein • Mitochondria • Apoptosis • SAS Tongue cancer cell*

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer and is known to have poor prognosis which is caused by local invasion and distant metastasis [1]. Although extensive research is being done on its pathogenesis and management, the 5-year survival rate for OSCC patients only improved to 53% over the past decades [2]. Chemotherapy is a standard and adjunctive treatment for surgery when tumor metastasis has involved the lymph nodes or distant organs, but the treatment has toxic side effects on many patients [3]. Numerous cancer research studies have focused on traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects compared with current chemotherapeutic agents.

Flavonoids, a large group of aromatic plant secondary metabolites that are produced from traditional Chinese medicinal plants, have shown the compelling data for the antitumor activities [4,5]. Extracts and isolated flavonoids from herbs could relieve oxidative stress and immune dysfunction associated with the onset and progress of cancer [6]. Studies have also demonstrated that flavonoids were able to arrest the cell cycle of tumor cell lines that are resistant to multiple

* *E-mail: xhjheb@163.com*

chemotherapeutic drugs and inhibit the progression of tumor angiogenesis [7,8].

The most versatile plants used as a source of flavonoids are *S. baicalensis* and *S. barbata*, the members of genus Scutellaria family. It has been reported that *S. baicalensis* and *S. barbata* have diverse biological activities that are anti-inflammatory [9], antioxidative [9], and antibacterial [10]. Recent investigations show that *S. baicalensis* and *S. barbata* alone, or in combination with other herbs, can inhibit cancer cell proliferation or induce apoptosis in breast [11], hepatocellular [12], lung [13], prostate [14], colon [15], and gynecologic neoplasms [16]. However, it is still unclear whether these activities are caused by the additive or synergistic effects of several components of *S. baicalensis* or *S. barbata*, or due to a single component of them.

Flavonoids, containing baicalein, baicalin, apigenin, scutellarein, scutellarin, wogonin, luteolin and apigenin, *etc.*, constitute the major phytochemical component of Scutellaria extracts. Preclinical studies have demonstrated that baicalein has a favorable effect in cisplatin-induced cell death of human glioma cells [17]. It also can induce apoptosis of myeloma cell lines via mitochondria pathway by inhibiting the phosphorylation of IkB-alpha [18]. Apigenin can inhibit growth of pancreatic cancer cells through suppression of cyclin B-associated cdc2 activity and G2/M arrest [19] and induce apoptosis of breast cancer cells through proteasomal degradation of HER2/neu [20]. Apigenin and luteolin induce apoptosis by down-regulating the Pharmacologica Sinica IGF-I expression, which contributes to the selective growth of leiomyoma [21]. Luteolin induces apoptosis of Lewis lung carcinoma cells by effectively activating caspase 9 and 3 and down-regulating the expression of extracellular signal-regulated kinase (ERK) and Akt [22]. Wogonin can inhibit phorbol 12-myristate 13-acetate-induced COX-2 gene expression in human lung epithelial cancer A549 cells [13] and promote the granulocytic differentiation of human promyelocytic leukemia cells by up-regulating the expression of phospholipid scramblase 1 gene [23]. Scutellarein, 5,6,7,4'-Tetrahydroxy flavone, is a flavonoid monomer composition of *S. baicalensis* and *S. barbata*. Its antitumor properties have rarely been studied. To explore the effects of scutellarein on the human tongue cancer SAS cells, we examined the effects it has on cells proliferation and apoptosis in vitro, and the morphology and function changes of mitochondria, and apoptosis-related protein expression of bcl-2, bax, and caspase 3 on mitochondrial pathways.

2. Materials and methods

2.1. Materials

Scutellarein (purity 99%, HPLC) was purchased from Guangzhou BiomolBio-Tec Co. Ltd. (Guangzhou, China). SAS human tongue cancer cell lines were from the Human Science Research Resources Bank (Osaka, Japan). RPMI-1640 and FBS were from Gibco BRL (Life Technologies, Paisley, Scotland). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO). MTT was from KeyGen (Nanjing, China). Annexin V-FITC apoptosis detection kit was from BD Biosciences (San Diego, CA). Antibodies against bcl-2, bax, caspase 3, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from Sigma (St. Louis, MO). Enhanced chemiluminesence (ECL) reagent was purchased from Amersham International (Amersham, UK). All other reagents and solvents used in experiments were of analytical grade.

2.2. Cell culture

Cells were maintained at 37°C in a humidified incubator (Heraeus, Germany) containing 5% CO2, in Roswell Park Memorial Institute 1640 (RPMI-1640) medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

2.3. MTT assay

Cells were plated at a density of 10,000 cells in 96-well plates. After incubation for 24 h, the cells were treated with different concentrations (1, 5, 10, 25 µM) of scutellarein for 24, 48,72 and 96 h. Drug cytotoxicity was evaluated by using a MTT assay. Fifty µl MTT was added to each well at 5 mg/ml, and incubation was continued for 4 h. Formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 150 µl DMSO and absorbance was measured at 490 nm using Stat Fax2100 microplate reader (Equl-Awaretech, USA). Each drug concentration was set up in six replicate wells, and the experiments were repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls.

2.4. Flow Cytometric Analysis

After treatment with different concentrations of scutellarein for 96 h, cells were collected and washed with cold

PBS. The cells were labeled by incubation with 5 mL FITC-annexin V and 10 mL PI at 250 mg/mL for 10 min in the dark at room temperature. The cells were washed with PBS again and examined by flow cytometry (BD Biosciences). Apoptosis was routinely quantified by counting the number of cells stained with FITC-labeled annexin V.

2.5. Transmission electron microscopy

After treatment with different concentration of scutellarein for 96 h, the cells were fixed in 40% glutaraldehyde solution and post fixed in 1% osmium for 2 h before they were immersed with Epon 821 for 96 h at 60°C. After that, the cells were sectioned into ultrathin slices (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was observed using TEM.

2.6. Mitochondrial depolarization assay

The cells treated with scutellarein for 96 h were incubated with an equal volume of JC-1 [5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide] staining solution (5 mg/ml) at 37°C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts or dual emissions from both mitochondrial JC-1 monomers and aggregates under an Olympus fluorescent microscope at 488 nm excitation. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio.

2.7. Western blot analysis

After treatment with different concentrations of scutellarein for 96 h, the cells were lysed by 100 µl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, TritonX-100 1%, EDTA 1 mM, and PMSF 2 mM) and incubated for 30 min on ice. The lysates were sonicated and centrifuged at 16,099g for 10 min, and the supernatants were collected and stored at -80°C before they were used in Western blot analysis. The protein concentrations were determined by the Bradford assay using bovine serum albumin as standard. Cells protein samples containing 20 µg of protein were separated by SDS-PAGE and electro blotted ontonitrocellulose sheets, blocked for 1 h at room temperature with a buffer (20 mM Tris , 150 mM NaCl, pH 7.6, 0.1% Tween 20) containing 5% nonfat dry milk and then probed with appropriate antibodies to bcl-2, bax, procaspase 3 andactin at 1:500 dilution overnight at 4°C followed by horseradishperoxidase-conjugated goat anti-rabbit IgG (1:50,00 dilution) or goat anti-mouse IgG (1:50,00 dilution) antibodies for 1 h at room temperature. Immunoreactivity was detected using ECL Western blotting detection kit and exposed to X-ray film. Immunoblots were scanned using a GS-800 densitometer and protein bands were quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.8. Statistical analysis

Experiments were independently performed at least 3 times. Data are presented as the mean ± SEM. Data were analyzed using one way ANOVA. Statistical significance was established at $P<0.05$.

3. Results

3.1. Scutellarein induce apoptosis of SAS cells

MTT assay was used to examine the effect of scutellarein on cell viability. As shown in Fig 1, scutellarein inhibited SAS cell viability significantly in a dose- and time-dependent manner when compared with the DMSO-treated control. The difference was significant for cells treated with 5 and 10 µM of scutellarein and incubated for 96 h, compared with the control group ($P<0.05$). Flow Cytometric analysis showed that the percentages of early and late apoptotic cells increased significantly after treatment with 5 and 10 µM scutellarein for 96 h (Fig. 2).

3.2. Effect of Scutellarein on cell morphology

To confirm the morphological changes of apoptosis, ultrastructural changes of SAS cells treated with various Scutellarein for 96 h were observed by TEM. The

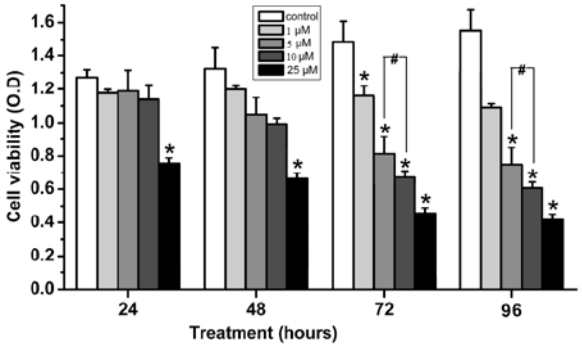

Figure 1. Scutellarein cytotoxicity on SAS cells. Cells were treated with various concentrations of scutellarein (1, 5, 10, 25 μM) and incubated for 24, 48,72 and 96 h. Scutellarin cytotoxicity was detected using MTT degradation assay, shown by absorbance at 490 nm. Data represent the mean ±SEM of three independent experiments. *$P < 0.05$ versus control, #$P < 0.05$ versus each other.

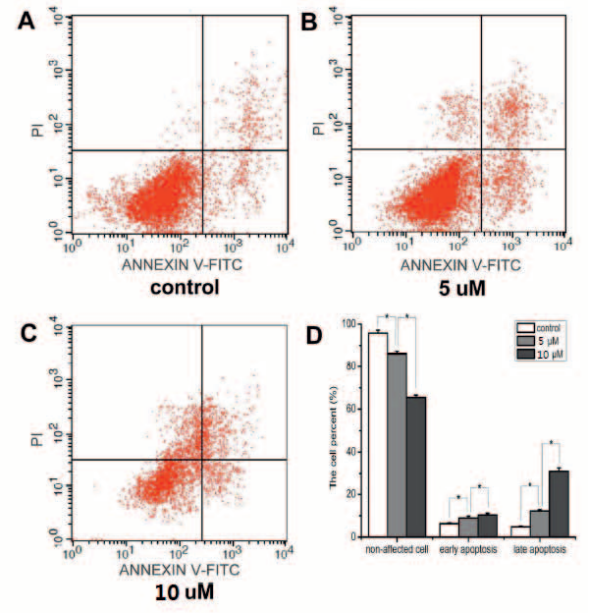

Figure 2. Effect of Scutellarein on the apoptosis of SAS cells. Apoptosis of SAS cells shown by annexin-V/PI staining after treated with 5 and 10 μM scutellarein for 96 h, with data showing the percentages of non-affected, early and late apoptotic cells. All values are given as mean ± SEM from three independent experiments. *P < 0.05 compared with each other.

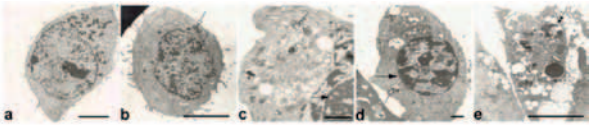

Figure 3. Effects of scutellarein on the ultrastructural changes of SAS cells. Cells were treated with various concentrations of scutellarein (5, 10 μM) and incubated for 96 h. The morphologic change of SAS Cells was detected by transmission electron microscopy. a, Control cells have large nucleus, rich chromatin and microvilli, and integral organelles. b, c: Nucleus shrunk (↟), chromatin condensation (↓), mitochondria swelled (↓) and even mitochondrial crest dissolved to form myeloid changes (↓) after treatment with 5 μM scutellarein. d, e: Microvilli defluxion, chromatin margination plaque, mitochondrial vacuolar degeneration (↓) and even apoptotic body (↓) formed after treatment with 10 μM scutellarein. a, b and e: Bar=5 μm; c and d: Bar=1 μm

control cells have large nuclei, are rich in chromatin and microvilli, and integral organelles (Fig.3a). Treated with 5 μM Scutellarein, the cells showed early morphological changes in apoptosis including nucleus shrinkage, chromatin condensation, mitochondrial swelling and mitochondrial myeloid degeneration (Fig. 3b and 3c). Microvilli loss, nuclear chromatin crescent-like clumps, vacuolar degeneration, nuclear membrane splitting, nuclei fragmentation, and apoptotic bodies were observed when cells were treated with 10 μM Scutellarein (Fig. 3d and 3e).

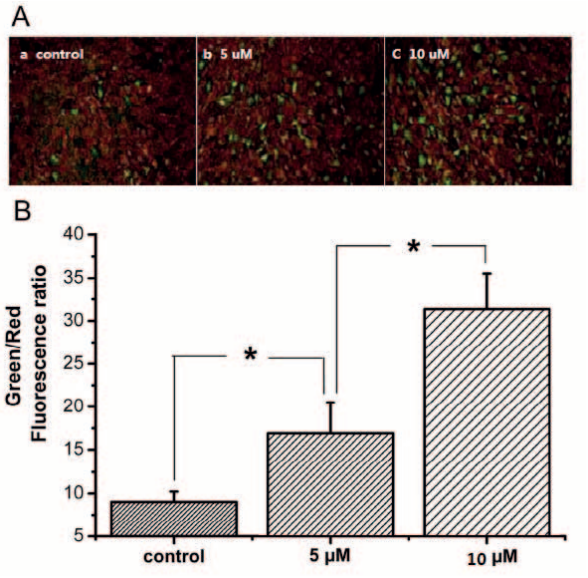

Figure 4. Effects of scutellarein on the ΔΨm of human tongue cancer SAS cells. SAS cells were treated with scutellarein (5, 10 μM) for 96 h and stained with JC-1 probe and imaged by a fluorescent microscope. The individual red and green average fluorescence intensities are expressed as the ratio of green to red fluorescence. The increase of fluorescence ratio, which is represented in the bars, is correlated with an increase in mitochondrial depolarization a-c. representative photographs of JC-1 staining in different groups. Quantitative analysis of the shift of mitochondrial green fluorescence to red fluorescence among groups. All values are denoted as mean ±SEM from ten independent photographs shot in each group. *P<0.05 compared with each other.

3.3. Scutellarein promoted mitochondrial depolarization

Mitochondrial function was assessed with the mitochondrial ΔΨm measured with JC-1 red fluorescence. Relative mitochondrial mass was determined by a fluorescent microscope using JC-1. The cells were stained with JC-1 probe for the measurement of mitochondrial depolarization. The quantitative analysis of JC-1-stained cells revealed a significant decrease in the red (high ΔΨm) to green (low ΔΨm) ratio in inscutellarein-treated cells when compared with control cells (Fig. 4A). The expression of Bcl-2 and Bax was associated with mitochondrial function. Western blotting assay showed that Bcl-2 expression in SAS cells was significantly suppressed after treatment for 96 h with 5 and 10 μM Scutellarein, showing a negative correlation with the Scutellarein dosage (Fig. 5A, 5B, 5D, n=3, p<0.05), while Bax expression was significantly increased, showing a positive correlation with the Scutellarein dosage (Fig. 5, n=3, p<0.05). The expression of caspase-3 was assayed for the purpose of confirming the apoptosis. As shown in Fig. 5, after treatment with Scutellarein for 96 h, the expression of caspase-3 remarkably increased

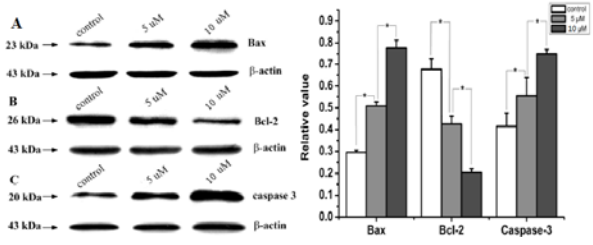

Figure 5. Effects of scutellarein on protein expression levels of Bax, Bcl-2 and Caspase-3 in SAS cells. SAS cells were treated with scutellarein (5, 10 μM) for 96 h. The protein expression levels of Bax, Bcl-2 and Caspase-3 were evaluated by western blotting assay. β-actin was used as an internal control. The expression levels of Bax and Caspase-3 increased as scutellarein concentration rose while the expression levels of Bcl-2 decreased compared to controls. Immunoblots were scanned using a GS-800 densitometer and protein bands from three independent experiments were quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA). All values were expressed as mean ± SEM. *P<0.05 compared with each other.

in a dose-dependent manner detected by western blot assay (Fig. 5, n=3, p<0.05). These results indicated that Scutellarein did induce apoptosis in a population of SAS cells.

4. Discussion

Apoptosis is required for correct development and organismal homeostasis, while malfunction of apoptosis contribute to the progression of cancer and degenerative diseases [24]. Because many chemotherapeutic drugs could induce apoptosis in malignant cells, apoptosis has currently been a target for developing antitumor drugs [25]. Recently, there is a growing interest in the use of natural materials or naturally occurring substances for the treatment or prevention of cancer because of the few side effects. This study shows that Scutellarein can inhibit human malignant glioma cell lineU87-MG and breast carcinoma MDA-MB-231 proliferation, but has no effect on human mammary epithelial cell and prostate cancer cell line PC3 [26]. Therefore, we suspected that the antitumor activity and mechanism of Scutellarein on tongue cancer might not be the same with other tumor cells.

In this study, we used MTT to verify the effect of scutellarein on SAS cell proliferation. Scutellarein was found to inhibit SAS cell viability significantly in a dose- and time-dependent manner (Fig.1) and Flow cytometric analysis indicated that the quantity of apoptotic cells increased with increasing concentrations of scutellarein (Fig. 2). Apoptotic cells are characterized by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation [27], which are the distinctive morphological features to confirm the apoptosis and its extent. SAS cells also exhibited morphological changes from early to late apoptosis when treated with different concentration scutellarein as detected by TEM (Fig. 3b-3e). These results indicate that the proliferation inhibition effect of scutellarein on SAS cells was due to the induction of apoptosis.

The ultra-morphological changes resulted from the gradual increase in mitochondrial swelling, and the mitochondrial crest was dissolved to promote myeloid changes and vacuolar degeneration when the concentration of scutellarein was increased from 5 and 10 μM (Fig. 3b-3d). Mitochondria are important intracellular organelles to supply energy and are regarded as the central regulator determining cellular survival and demise [28]. The mitochondrial structure changed significantly when cell apoptosis occurred. Mitochondrial cristae may lose packed and ordered folding. The membrane gap is widened and filled with concentrated Matrix. Mitochondria appear to have extensive swelling and have vacuolar degeneration [29].

Mitochondrial dysfunction is characterized by the loss of $\Delta\Psi m$ which is an early event in the cell apoptotic process. $\Delta\Psi m$ was decreased before chromatin condensation and DNA fragmentation. Reduced $\Delta\Psi m$ induces Cyt-c release from the mitochondria, and causes apoptosis [30]. In this study, scutellarein significantly decreased the $\Delta\Psi m$ (Fig. 4), suggesting that scutellarein induced apoptosis of SAS cells via the mitochondrial pathway. Loss of $\Delta\Psi m$ was found to be closely associated with the expression of Bcl-2and Bax. The Bcl-2 family protein, localized to the outer mitochondrial membranes, controls the stabilization of mitochondrial membranes (Fig. 5). The expression of Bax was positively correlated with decreased $\Delta\Psi m$ and the expression of Bcl-2 was negatively correlated with decreased $\Delta\Psi m$, suggesting that increased Bax expression or decreased Bcl-2 expression may be involved in the decreased $\Delta\Psi m$. Increased Bax/Bcl-2 proportion will lead to Cyt-c release from the mitochondria, thus inducing cell apoptosis [31]. *S. barbata* might induce tumor cells apoptosis by suppressing the expression of bcl-2 or increasing the ratio of bax/bcl-2 [32].

The extrinsic pathway and the intrinsic pathway are two major pathways leading to cell apoptosis. Caspase family is central to the proteolytic events of apoptosis and it can be activated by an apoptosis death stimulus. Activation of the initiator of caspases (procaspases 8-10) led to proteolytic activation of downstream effector caspases (caspase-3, -6, -7). In particular, the activation of caspase-3 is a common event in both pathways. As detected in many apoptotic tumor cells induced by *S. barbata* [33], our studies also showed that caspase 3

was activated (Fig. 5). It indicated that exogenous scutellarein induces apoptosis of SAS cells.

In summary, scutellarein can up-regulate the expression of Bax and down-regulate the expression of Bcl 2, promote mitochondrial depolarization and damage mitochondrial structure, and induce SAS cells apoptosis. It implies that scutellarein can induce apoptosis of tongue cancer SAS cells in vitro through activating the mitochondria pathway. Its effectiveness demonstrated here suggests that scutellarein may be a promising chemotherapy agent in inhibiting tumor growth.

Acknowledgements

This work was supported the Scientific Research Foundation of Heilongjiang Provincial health Department (2009-094).

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