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The potential anticancer activity of extracts derived from the roots of *Scutellaria baicalensis* on human oral squamous cell carcinoma cells

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Abstract. Various herb products derived from plants have potent biological effects including anticancer activity. In the present study, the antitumor activity of a herbal product derived from the *Scutellaria baicalensis (S. baicalensis)* was examined, using *in vitro* assays in a human oral squamous cell carcinoma (OSCC) cell line. Results showed that *S. baicalensis* root extract at the concentration of 100 μg/ml inhibited monolayer- and anchorage-independent growth in human OSCC cell lines, while not affecting the adhering abilities of cells. This suggested that it did not alter the expression of any of the adhesion receptors that mediate cell-extracellular matrix (ECM) interactions. The *S. baicalensis* root extract demonstrated potent cytostatic and apoptotic effects due to the downregulation of the cyclin-dependent kinase 4 expression and its partner cyclin D1, resulting in G1 arrest and poly (ADP-ribose) polymerase (PARP) cleavage. Additionally, the *S. baicalensis* root extract was found to have blocked vascular endothelial growth factor (VEGF)-induced migration and tube formation in human endothelial cells. Taken together, these results demonstrate that as a herbal product, the *S. baicalensis* root extract is a potential inhibitor of tumori- and angiogenesis and may be valuable in the development of pharmaceutical medications for the treatment of oral squamous cell carcinoma.

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Introduction

Squamous cell carcinoma of the head and neck is the sixth most common cancer worldwide, with approximately 600,000 patients developing this type of cancer annually (1). Despite recent advancements in cancer treatment, the overall survival of patients with oral squamous cell carcinoma (OSCC) has not improved significantly over the past 20 years. Cancer chemotherapy is an important alternative to surgery and radiation in treating OSCC. However, although the conventional anticancer medications are effective and useful, the resistance and toxic responses of patients to these medications remain a problem.

Herbal products contain a wide variety of chemical compounds with potent biological effects, including anticancer activity (2-5). Identification of the active components in herbal products and their mechanism of action is of ever-growing interest in the field of pharmaceutical medication for clinical use development worldwide. In 2010, our department initiated a collaborative project aiming to identify new chemopreventive and chemotherapeutic agents in natural products with the Tokyo University of Marine Science and Technology, having provided more than 400 bio-active herbal products. Subsequent to the examination of these products by cytotoxic and growth inhibition assays, *Scutellaria baicalensis (S. baicalensis)* root extract demonstrating a potential anticancer activity was thoroughly examined.

Traditionally, the dried roots of *S. baicalensis* have been used in the Chinese herbal medicine 'Huang Qin' to treat a variety of diseases including viral hepatitis, inflammatory diseases and bacterial infections (3,6,7). The *S. baicalensis* root extract contains three major flavonoids: baicalin, baicalein and wogonin. In previous studies, these flavonoids were shown to have the potential to arrest certain tumor cell lines, while inhibiting tumor angiogenesis (8-11). However, the anticancer effects of this compound, including the crude root extract, havenever been studied in the framework of OSCC. The present study aimed to investigate the effects of the *S. baicalensis* root extract on OSCC cell proliferation, cell-cycle progression and apoptosis.

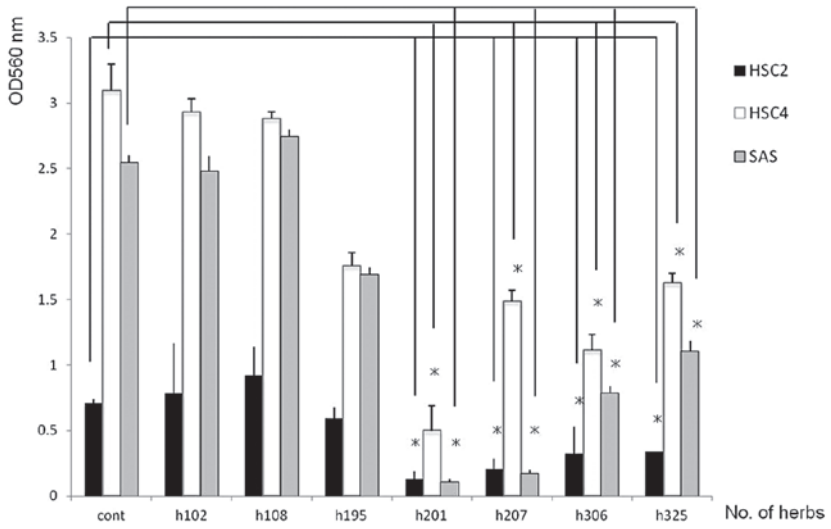

Figure 1. Examination of herbal products for potent anti-proliferative activities on human OSCC cell lines is shown. At 24 h after seeding, OSCC cells were treated with various concentrations of herbal products (h102, h108, h195, h201, h207, h306 and h325) and cultured for 3 days.The herbal product h201 exhibited a potent inhibition of monolayer proliferation. Asterisks indicate statistically significant differences vs. control at $^{*}P<0.05$. OSCC, oral squamous cell carcinoma.

Materials and methods

Preparation of Scutellaria baicalensis and the reagents. The roots of *S. baicalensis* were collected and imported from China. A specimen was deposited in the herbarium of the Tokyo University of Marine Science and Technology. Dry powdered roots (100 g) of *S. baicalensis* were extracted and then concentrated until dry at 1 mg/ml under reduced pressure. Cis-diamminedichloroplatinum [(CDDP) brand name, Randa®] was purchased from Nippon Kayaku (Tokyo, Japan). CDDP was used as the positive control in each *in vitro* assay system (proliferation and DNA fragmentation assay), since CDDP is the most promising antitumor (antiproliferative and apoptosis-inducing) medication for the treatment of OSCC.

Cell culture and hypoxic condition. OSCC cell lines (HSC2,3,4, SAS) derived from a human oral squamous cell carcinoma were cultured at 37˚C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) derived from human umbilical cords were purchased from Lonza Biologics (Basel, Switzerland) and used between passages 3 and 7. These cells were cultured in endothelial cell basal medium (EBM)-2 complete medium (Lonza Biologics). Hypoxia experiments were performed for the indicated times in a humidified INVIVO$_2$ workstation (Ruskinn Technology, South Wales, UK), calibrated to deliver 5% CO_2, 2% O_2 and 93% N_2 at 37˚C.

Proliferation assay. Monolayer- and anchorage-independent growth assays were performed as previously described (12). The monolayer cell proliferation was measured using an MTT assay kit (Roche Diagnostics Corp., Indianapolis, IN, USA) that measures a purple formazan compound produced by viable cells (13). The anchorage-independent growth was measured using a commercial kit, CytoSelect™ 96-Well *In Vitro* Tumor Sensitivity Assay (Cell Biolabs, Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Extracellular matrix (ECM)-related assay. Adhesion assays were carried out using the CytoSelect™ 48-Well Cell Adhesion Assay (Cell Biolabs, Inc.). SAS cells ($1x10^5$) were seeded onto the wells and were incubated at 37˚C for 60 min in a tissue culture incubator prior to washing and staining. Gelatin zymography was performed according to the method described previously (14). SAS cells were cultured as described in the 'Collection of conditioned medium' subsection, and the conditioned medium was harvested and lyophilized. Equivalent amounts of samples were dissolved in a Tris-HCl buffer containing 30% glycerol, 7.7% SDS and 0.3% bromophenol blue at pH 6.8.

Collection of conditioned medium. Prior to use in experiments, HUVECs were maintained in an EBM-2 medium without hydrocortisone for at least 24 h. The removal of hydrocortisone was necessary, since it inhibits metalloproteinase production. Once passed and plated, endothelial cells grew normally even in the absence of hydrocortisone.

The SAS cells were inoculated at a density of $1x10^6$ cells in 10-cm diameter dishes and cultured for 60 h in DMEM containing 10% FBS. The cells were replenished with fresh medium and cultured for 24 h. The cells were then replenished with fresh medium and placed under normoxic or hypoxic conditions for 12 h. The cell culture supernatant and the cell layer fraction were harvested for subsequent analysis.

Western blot analysis. Western blot analysis of fraction markers was carried out, according to the method described in a previous study (15). Anti-phospho-p42/p44 mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase (ERK)1/2] and anti-phospho-p38 were obtained from Promega (Madison, WI, USA), while anti-p42/p44 MAPK (ERK1/2), anti-phospho-c-Jun NH2-terminal kinase and anti-c-Jun NH_2-terminal kinase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-p38 obtained from Calbiochem (Bad Soden, Germany). CDK4, CDC2, and anti-Bcl-2 antibodies and peroxidase-conjugated

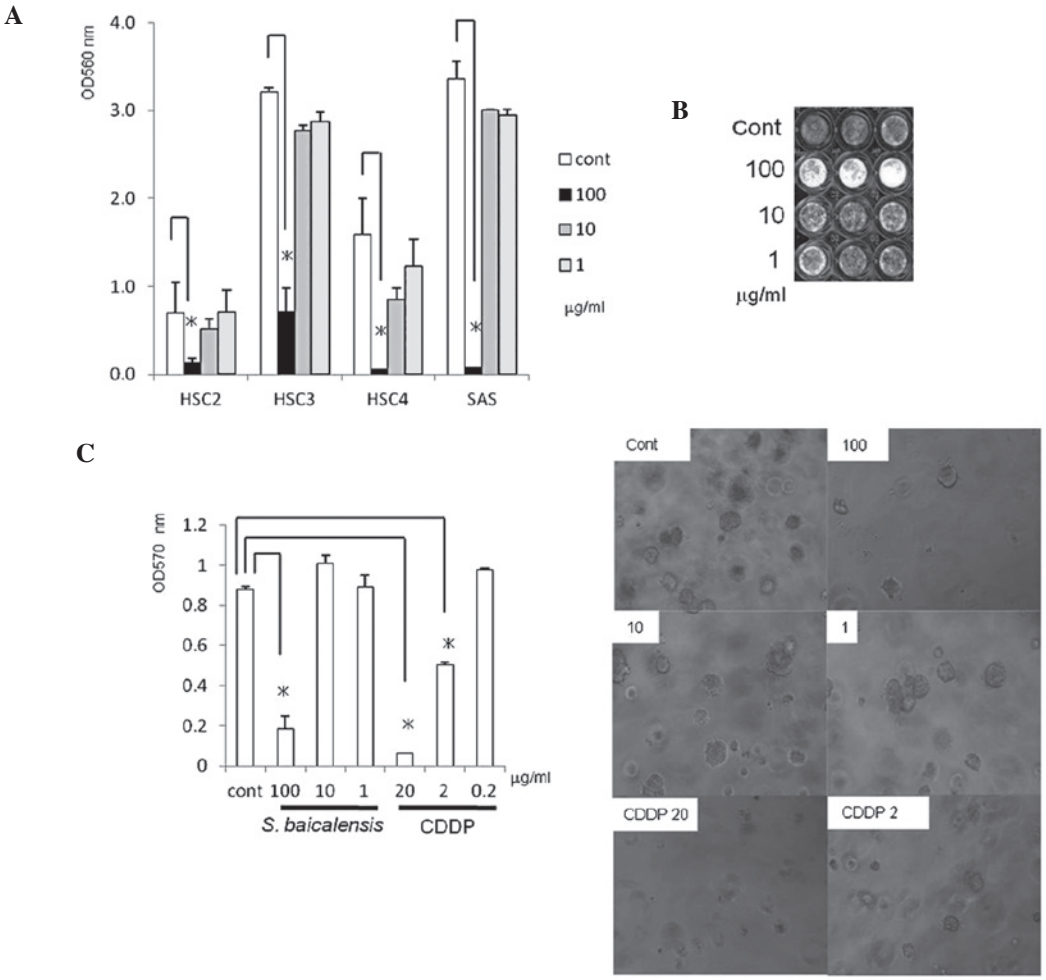

Figure 2. Effect of *Scutellaria baicalensis (S. baicalensis)* on the growth of OSCC cells is shown. (A) OSCC cells were treated with various concentrations of *S. baicalensis* and cultured for 3 days. *S. baicalensis* extract at a dose of 100 μg/ml significantly inhibited monolayer proliferation. Asterisks indicate statistically significant differences vs. the control at *P<0.05. (B) Potent inhibition of the proliferation by *S. baicalensis* at 100 μg/ml was observed in SAS cells using crystal violet stain. (C) Concentration-dependent effects of *S. baicalensis* on the morphology of SAS cells is shown. Cells incubated for 5 days with or without *S. baicalensis* as described in Materials and methods were examined under a microscope and photographed using a digital CDD camera. Asterisks indicate statistically significant differences vs. the control at *P<0.05. CDDP, cis-diamminedichloroplatinum-exposed samples were used as the positive control.

secondary antibody were purchased from Santa Cruz Biotechnology, Inc.

DNA fragmentation analysis. DNA fragmentation electrophoretic analysis and terminal deoxyuridine nick-end labeling (TUNEL) assay were carried out as described previously (15). Cells were observed at 24 h subsequent to treatment with *S. baicalensis* root extract for the two assays.

Tube formation analysis. Tube formation of endothelial cells was performed as described previously (16). Matrigel (50 μl) was pipetted into 96-well dishes and then polymerized. HUVECs incubated in EBM-2 medium without vascular endothelial growth factor (VEGF) and hydrocortisone for 6 h were harvested subsequent to trypsin treatment, while various concentrations of *S. baicalensis* were added to the cells prior to their being seeded and plated onto a layer of Matrigel at a density of $3x10^5$ cells/well, followed by the addition of 10 μg/ml VEGF. The cultures were photographed after 12 h (x10, x40 magnifications).

Statistical analysis. Unless otherwise specified, the experiments were repeated at least twice, with similar results. Statistical analysis was carried out using the Student's t-test. Data were shown as the means ± standard deviations. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of S. baicalensis root extracts *on the growth of several human oral squamous cell carcinoma cell lines*. According to a series of screening analyses using MTT assays, seven herbal products (h102, h108, h195, h201, h207, h306 and h325) were available out of >400 bio-active herbal products. In the cell lines tested, h201 induced significant growth inhibition at a dose of 100 μg/ml compared to other herb products (Fig. 1). The active compound in the h201 herbal product was identified as the *S. baicalensis* root extract.

Effects of S. baicalensis on the growth of OSCC cells. The *S. baicalensis* root extract inhibited monolayer- and

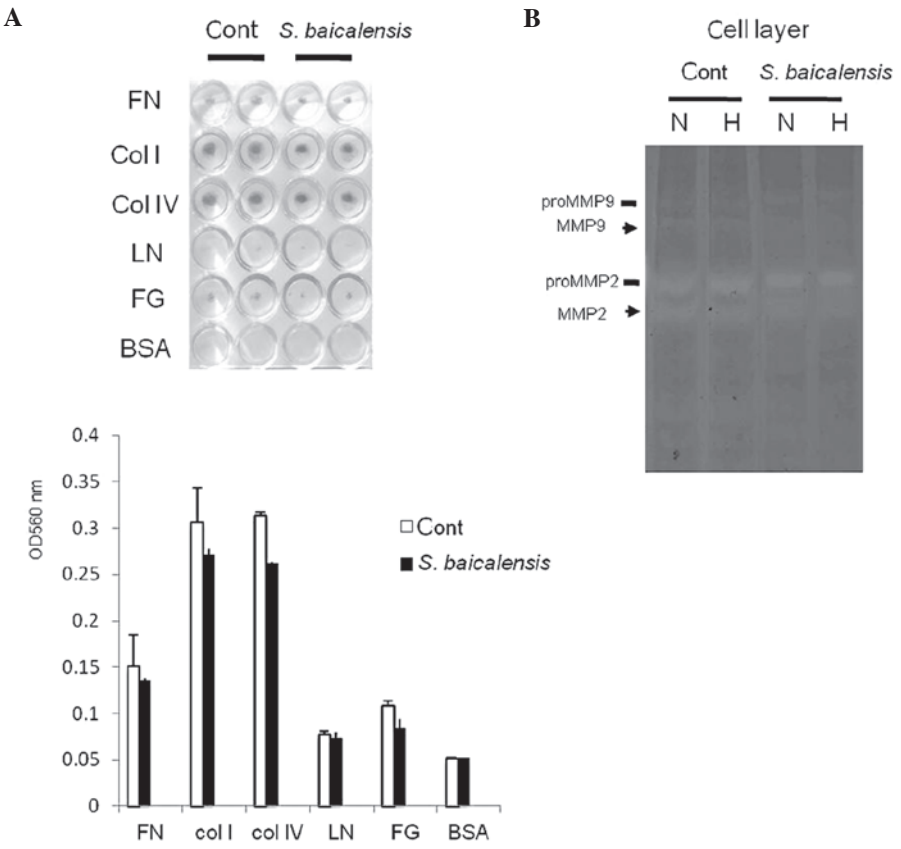

Figure 3. Effect of *Scutellaria baicalensis* (*S. baicalensis)* on the ECM adhesion and proteolytic activities in SAS cells is shown. (A) *S. baicalensis* extract at a dose of 100 μg/ml had no effect on SAS cell adhesion to ECM. FN, fibronectin; Col I, collagen I; Col IV, collagen IV; LN, laminin I; FG, fibrinogen; BSA, bovine serum albumin. (B) The levels of proteolytic activities and matrix metalloproteinase (MMP)-2 and -9 in SAS cells exposed to 100 μg/ml of *S. baicalensis* in both hypoxic (H) and normoxic (N) conditions for 12 h were examined. Pro-MMP, pre-proenzyme MMP.

anchorage-independent growth in OSCC cells. To examine the growth-inhibitory activity of *S. baicalensis* extracts in several human OSCC cell lines, additional MTT assays were performed at various concentrations of the *S. baicalensis* extract (1-100 μg/ml). The *S. baicalensis* extract at a dose of 100 μg/ml showed significant inhibition of monolayer proliferation in cells from the four cell lines (Fig. 2A). Similar results were obtained in SAS cells using crystal violet staining (Fig. 2B). The *S. baicalensis* extract was also able to markedly inhibit anchorage-independent growth at the same dose (Fig. 2C).

Effect of S. baicalensis on the ECM adhesion and proteolytic activities in SAS cells. The *S. baicalensis* root extract did not affect adhesion to ECM despite its proteolytic activities of matrix metalloproteinases in SAS cells. Given that in order to metastasize, malignant tumor cells require several distinct cellular functions including migration, adhesion, detachment and ECM proteolysis, the ECM adhesion and the proteolytic activities of cells incubated with 100 μg/ml of *S. baicalensis* were then examined using a cell adhesion assay and gelatin zymography. *S. baicalensis* had no effect on cell adhesion to ECM (Fig. 3A). Hypoxia converted proMMP-2 into its active form in SAS cells, whereas the levels of proteolytic activities detected at a dose of 100 μg/ml of *S. baicalensis* were inhibited in the hypoxic-SAS cell samples compared to normoxic ones (Fig. 3B).

Effect of S. baicalensis on the activation of three subgroups of MAPKs. To understand the molecular mechanism whereby *S. baicalensis* inhibits cell proliferation, initially the effects of *S. baicalensis* on signal transduction were investigated. As a result, *S. baicalensis* at a dose of 100 μg/ml induced the phosphorylation of ERK as early as at 30 min (Fig. 4).

S. baicalensis induces apoptotic cell death. To investigate the molecular mechanism underlying the observed growth-suppressing effects of *S. baicalensis*, assays were conducted to detect the induction of cell apoptosis in *S. baicalensis*-treated SAS cells. G1/S-transition proteins (CDK4 and its partner cyclin D1) were depleted within 6 h subsequent to stimulation by *S. baicalensis* (Fig. 5A). Furthermore, an increase in the cleaved poly (ADP-ribose) polymerase (PARP) expression was observed at 12-24 h. The apoptosis-related protein Bcl-2 expression also decreased markedly, within 12-24 h. DNA fragmentation was observed in SAS cells treated with *S. baicalensis* at a dose of 100 μg/ml (Fig. 5B). Apoptosis was directly confirmed by a significant increase in TUNEL-positive cells (Fig. 5C).

S. baicalensis inhibits VEGF-induced proliferation and tube formation of endothelial cells. To determine the anti-angiogenic activity of *S. baicalensis in vitro*, its inhibitory effect on the VEGF-induced proliferation of endothelial cells was evaluated. VEGF (10 ng/ml) significantly increased proliferation of

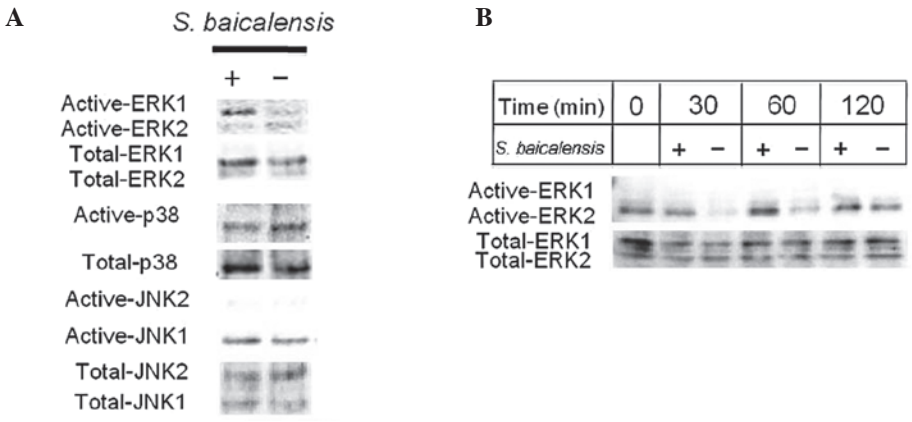

Figure 4. Signal transduction profile in sanguinarine-treated SAS cells is shown. (A) SAS cells were cultured subsequent to stimulation by *S. baicalensis* (100 μg/ml) for 60 min, after which phosphorylated or total ERK1/2, phosphorylated or total p38 and phosphorylated or total c-Jun NH_2-terminal kinase 1/2 (*JNK1*/*JNK2*) were analyzed by western blot analysis. (B) SAS cells were cultured after stimulation by *S. baicalensis* (100 μg/ml) for the indicated times, after which phosphorylated or total ERK1/2. Phosphorylated cells were analyzed by western blot analysis.

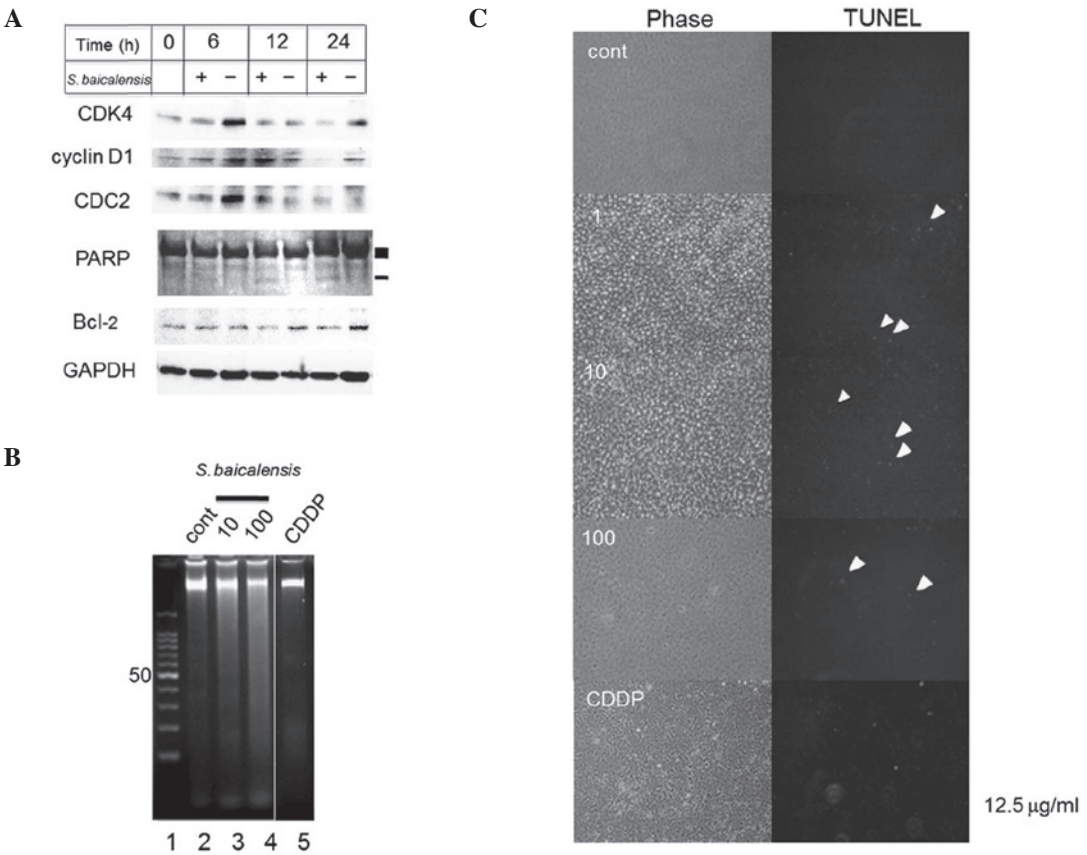

Figure 5. Cell cycle and pro-apoptotic effect of *S. baicalensis* in SAS cells are shown. (A) Immunoblotting analysis of G1/S (CDK4, cyclin D1), G2/M (CDC2) cell cycle transition proteins and apoptotic-related protein (Bcl-2, PARP: ■, full length and -, cleaved fragment) expression. (B) The DNA fragmentation was analyzed by gel electrophoresis, as described in Materials and methods. Lane 1, DNA marker; lane 2, normal sample; lanes 3 and 4, *S. baicalensis*-exposed samples; lane 5, CDDP-exposed samples (12.5 μg/ml) is the positive control. (C) TUNEL staining is shown. SAS cells were treated with either *S. baicalensis* (1, 10 or 100 μg/ml) or the vehicle for the indicated periods, then were observed by phase-contrast microscopy (Phase) and fluorescence TUNEL staining (TUNEL). Arrowheads indicate typically apoptotic cells showing the concentration or fragmentation of nuclei.

HUVECs, resulting in complete blockage by *S. baicalensis* (100 μg/ml) (Fig. 6A and B). When kept in a EBM-2 medium without hydrocortisone and VEGF, and then placed on Matrigel in the presence of VEGF, HUVECs were induced by VEGF to form elongated and robust tube-like structures organized by a larger number of cells, compared to the control. *S. baicalensis* effectively abrogated the tube formation induced by VEGF in a concentration-dependent manner (Fig. 6C).

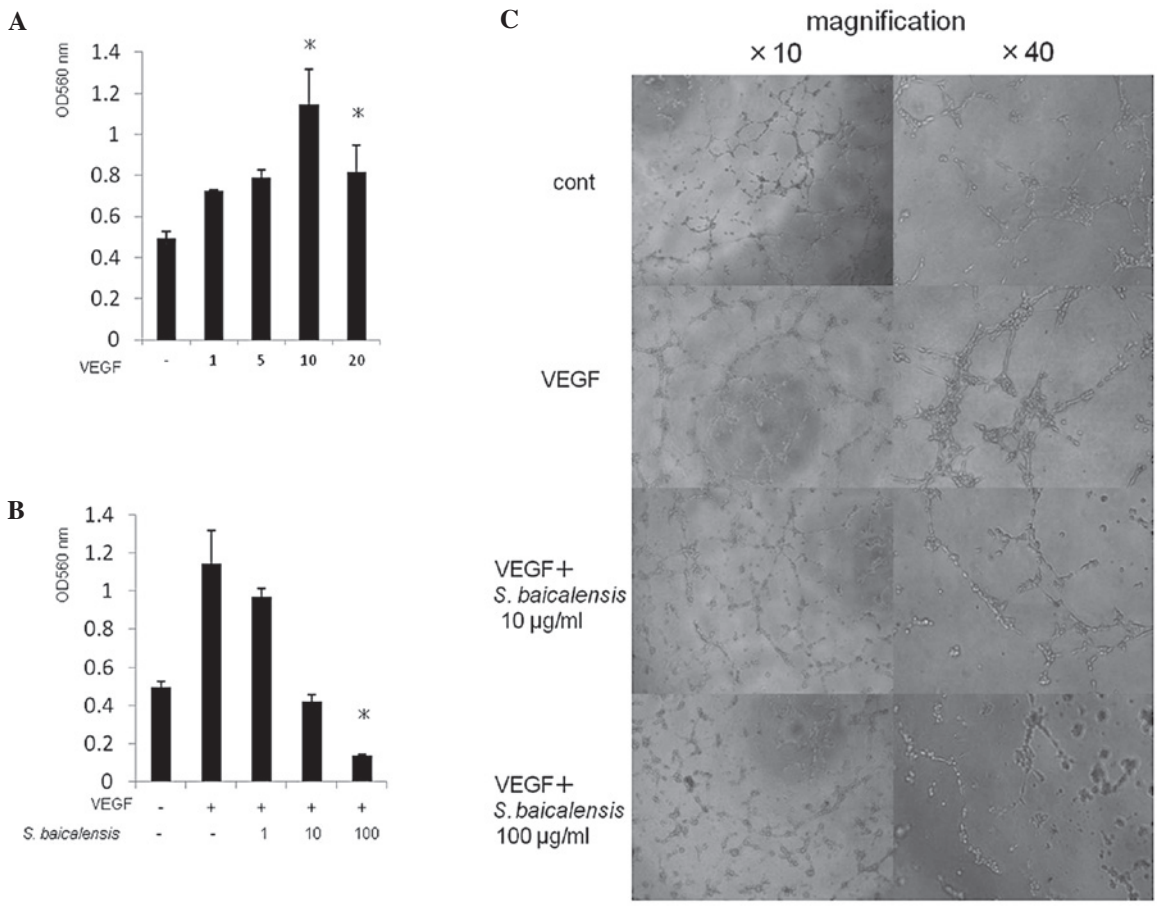

Figure 6. *Scutellaria baicalensis* (*S. baicalensis*) inhibits the VEGF-induced proliferation and tube formation of human umbilical vein endothelial cells. (A) HUVECs were treated with various concentrations of VEGF and cultured for 3 days. Asterisks indicate statistically significant differences vs. the control at $^{*}P<0.05$. (B) HUVECs were pre-treated with various concentrations of *S. baicalensis*, then cells were stimulated with 10 ng/ml VEGF and allowed to proliferate for 3 days. Asterisks indicate statistically significant differences vs. the control at $^{*}P<0.05$. (C) HUVECs were pre-treated with various concentrations of *S. baicalensis* (10 or 100 μg/ml) prior to treatment with 10 μg/ml VEGF. Representative endothelial tubes are shown. at a magnification of x10 and x40.

Discussion

Oral cancer treatment essentially aims to identify chemopreventive and therapeutic agents that selectively target OSCC cells without cytotoxic effects on healthy cells and tissues. Although there are various chemotherapeutic agents used to treat OSCC, treatment with currently existing chemotherapeutic medications, such as CDDP and 5-fluorouracil (5-FU) does not always substantially induce a positive response, since tumor cells are not dependent on a single receptor or signal transduction pathway for growth and progression. Therefore, finding new compounds demonstrating anticancer effects on OSCC is of utmost importance. These compounds may provide new and more effective treatment regimens for the treatment of OSCC (17-21).

In the present study, we examined a large number of herbal products and found the *S. baicalensis* extracts to have potent anti-proliferative and apoptotic effects on human OSCC cell lines. While most of the previous studies on *S. baicalensis* have focused on severe acute respiratory syndrome (SARS) (22-24), additional studies have demonstrated that flavonoids such as baicalin, baicalein and wogonin derived from *S. baicalensis* showed antitumor and anti-angiogenic effects on various cancer cell lines (25-27). By contrast, the effects of these flavonoids are not anti-proliferative in all the cell lines, suggesting that they exert either pro- or anti-apoptotic activity, depending on the cell type. The anti-proliferative and apoptogenic properties of the *S. baicalensis* root extract on OSCC cell lines may be attributed to the pro-apoptotic activity of these flavonoids at a late stage. A postulated mechanism for this is the inhibition of cell cycle regulatory proteins (CDK4, cyclin D1) within 6 h (Fig. 5A), preventing the release of E2F to mediate the G1-to-S transition, thus leading to a potent cytostasis, despite the potential of the *S. baicalensis* root extract to induce ERK, a survival protein, phosphorylation at an early stage (as early as at 30 min) (Fig. 4B).

Despite the expectations, combined evidence drawn from the ECM-related assay suggested that *S. baicalensis* does not affect the expression of any of the adhesion receptors, such as integrin, which mediates cell-ECM interactions despite having an effect on MMP-2-associated proteolytic activity. The cascade of invasion and metastasis through adhesion molecules or protease activity on OSCC cells is likely to be affected in part by *S. baicalensis*.

Angiogenesis is a useful target of cancer treatment, since most angiogenesis inhibitors specifically target the newly formed vasculature in tumors, as opposed to the quiescent blood vessels, thus reducing the possibility of side effects (16).

At a cellular level, *S. baicalensis* completely suppressed the stimulatory effect of VEGF on endothelial cell tube formation, suggesting that *S. baicalensis* suppresses angiogenesis via the differentiated inhibition of human endothelial cells. These data have shown that the *S. baicalensis* strategy has dual molecular properties through the prevention of tumor-induced angiogenesis and the direct cytotoxic effect on tumor cells. Although the half-maximal inhibition was relatively high, the potential properties of *S. baicalensis* leading to the blockage of OSCC tumor blood supply are likely to be beneficial for future chemopreventive and therapeutic agents used against this disease.

Whether or not flavonoids derived from *S. baicalensis* are useful in the chemoprevention and/or chemotherapy of human OSCC cells has yet to be determined. At present, additional studies are being conducted aiming to identify the active flavonoid component of the extracts, as well as the precise molecular mechanism whereby the flavonoid-induced inhibition of the human OSCC cell growth in mice bearing OSCC cell xenografts occurs.

In conclusion, based on the data provided in the present study, *S. baicalensis* is suggested to induce cytostasis and apoptosis in human OSCC cells. Consequently, *S. baicalensis* might be a promising chemopreventive and therapeutic agent for the treatment of OSCC.

Acknowledgements

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