The Apoptotic Effect of the Hexane Extract of *Rheum undulatum* L. in Oral Cancer Cells through the Down-regulation of Specificity Protein 1 and Survivin

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The hexane extract of *Rheum undulatum* L. (HERL) has been shown to have anti-cancer activity in several cancers *in vivo* and *in vitro*. However, the anti-cancer activity of HERL and its molecular mechanism in human oral cancer cells has not been explored. Thus, the aim of this study was to elucidate the growth-inhibitory and apoptosis-inducing effects of HERL in HN22 and SCC15 oral cancer cell lines. This study shows that HERL inhibits oral cancer growth, decreases cell viability, and causes apoptotic cell death in HN22 and SCC15 cells, as characterized by morphological changes, nuclear condensation and fragmentation, the cleavage of PARP and the accumulation of cells in the sub-G_1 phase. The treatment of oral cancer cells with HERL also resulted in decreased expression of specificity protein (Sp1) and its downstream protein, survivin. Therefore, our results suggest that the regulation of Sp1 and survivin plays a critical role in HERL-induced apoptosis in human oral cancer cells.

Keywords: Hexane extract of *Rheum undulatum* L. (HERL), apoptosis, specificity protein 1, survivin, oral cancer

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Oral and pharyngeal cancer is the sixth most common cancer world-wide and two-thirds of these cases occur in developing countries (Parkin *et al*, 2005). Despite advances in cancer diagnosis and treatment, there has been little improvement in the 5-year survival rate of oral cancer patients in the last few decades. Because of this, various approaches have been used in the clinical treatment of oral cancer patients in the last three decades, from non-targeted chemotherapy to highly targeted pharmacological inhibitors and specific monoclonal antibodies (Hamakawa *et al*, 2008; Scully and Bagan, 2009). Although targeted therapies yield better outcomes than non-targeted therapies, frequent treatment failure suggests the need for new strategies for this disease, including new drugs or targets.

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Rheum undulatum L. (RL), a well-known traditional Chinese medicine, has been widely used for thousands of years in China for the treatment of many diseases (He *et al*, 2009). In particular, the root of the species is widely used as a purgative and anti-inflammatory agent in East Asia (Chang *et al*, 1996; Kuo *et al*, 2001; Yu *et al*, 2008). It has been also reported that RL is used traditionally in Korea for the treatment of dental disease (Kim *et al*, 2010). Recently, it was found that RL contains many anthraquinones and their glycosides, including aloe-emodin, rhein, emodin, and chrysophanol, which are thought to be the major active components for inhibiting tumor growth in various cancer cells (Cha *et al*, 2005; Huang *et al*, 2006; Huang *et al*, 2007; Dorsey and Kao, 2007). However, there has been no study performed to investigate the effect of RL on the growth of human oral cancer cells.

In the present study, we examined the effect of the hexane extract of *Rheum undulatum* L. (HERL) on the growth of oral cancer cells and the molecular targets for HERL-induced apoptosis. The results demonstrated that HERL induced apoptotic cell death to inhibit the proliferation of oral cancer

cells and the down-regulation of Sp1 and survivin mediated the anti-cancer activity of HERL.

Materials and Methods

Reagents

The antibodies for poly(ADP-ribose) polymerase (PARP) and survivin were obtained from BD Biosciences (San Jose, CA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. Antibodies for Sp1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4'-6-Diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were acquired from Sigma-Aldrich (St. Louis, MO, USA). HERL was kindly supplied by professor Jeon JG (Jeonbuk National University, Jeonju, Korea).

Cell culture and chemical treatment

The HN22 and SCC15 human oral cancer cell lines were obtained from the School of Dentistry, Dankook University (Cheonan, Korea). The cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/mL streptomycin (WelGENE, Daegu, Korea) in a humid atmosphere of 5% CO_2. Equal numbers of cells were seeded and allowed to attach overnight. The cells were treated with 0.1% NaCl or HERL (20, 40 or 60 μg/mL) diluted in DMEM with 5% FBS for 48 h.

MTS assay

The effects of HERL on cell viability were estimated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were seeded in 96-well plates and incubated with various concentrations of HERL. After the treatment with HERL for 48 h, 30 μL of 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) (MTS) solution was added to each well and the cells were incubated for 2 h at 37°C. MTS solution was analyzed using a microplate reader (BioTeck Instruments, Winooski, VT, USA) at 490 and 690 nm (background).

FACS analysis for sub–G_1 DNA measurement

After treatment with HERL for 48 h, the detached HN22 and SCC15 cells were collected and combined with the adherent cells that had been released by trypsinization. The cells were fixed in 70% ethanol overnight at −20°C. The cells were subsequently stained with 0.02 mg/mL PI. The data were acquired using a FACScan flow cytometer.

DAPI staining

The number of cells with nuclear condensation and fragmentation was measured using DAPI staining. After treatment with HERL or 0.1% NaCl (control), the HN22 and SCC15 cells were harvested by trypsinization. The cells were resuspended in PBS, deposited on poly-$_L$-lysine-coated slides, stained with DAPI solution (2 μg/mL) and observed under a fluorescence microscope.

Western blot analysis

After treatment with HERL, HN22 and SCC15 cells were harvested. The protein supernatant fractions were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% skim milk followed by hybridization with the indicated antibodies. After incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody, the immunoreactive protein bands were observed using a chemiluminescence detection kit.

Statistical analysis

Data were assessed for statistical significance using a Student's *t*-test. A value of $P<0.05$ compared to the vehicle control was considered statistically significant.

Results

HERL decreases the cell viability in oral cancer cell lines

We first investigated the effect of HERL on the growth of HN22 and SCC15 cells. HERL-treated cells were detached from the dishes compared to 0.1% NaCl-treated cells and these morphological changes occurred in a dose-dependent manner (Figure 1A). We also measured the effect of HERL on cell viability in the MTS assay. The results showed dose-dependent decreases of cell viability in HN22 and SCC15 cells (Figure 1B). These results suggest that HERL has a growth-inhibitory effect on HN22 and SCC15 cells.

HERL induces apoptosis in oral cancer cell lines

To examine whether HERL induced apoptosis, the number of cells accumulated in the sub-G_1 phase of the cell cycle was counted by flow cytometric analysis. As shown in Figure 2, the sub-G_1 population of HERL-treated cells accumulated in a dose-dependent manner compared to the vehicle control. To confirm the induction of apoptosis by HERL in HN22 and SCC15 cells, DAPI staining was performed and showed the fragmentation and condensation of nucleus in the cells treated with HERL (20, 40 and 60 μg/mL) for 48 h compared
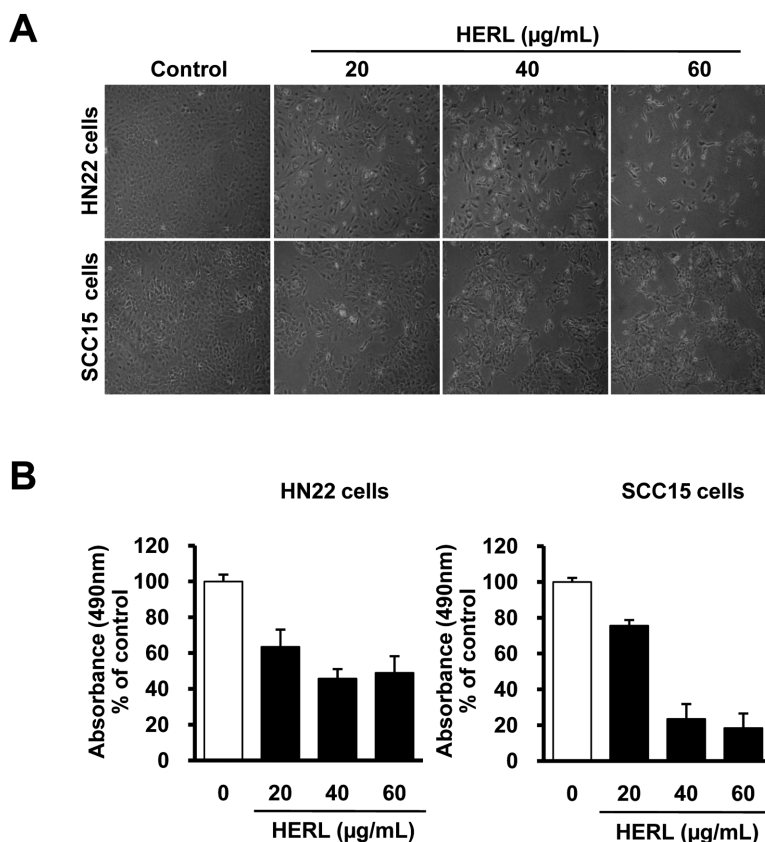
A

B

Figure 1. Morphological changes and the viability of HN22 and SCC15 cells after treatment with hexane extract of *Rheum undulatum* L. (HERL). A, Photomicrographs of HN22 and SCC15 cells treated with 20, 40 and 60 µg/mL of HERL for 48 h. B, Cell viability was determined using MTS assay. The graph was representative of three independent experiments and bar is mean±SD. *P<0.05 compared to control group.
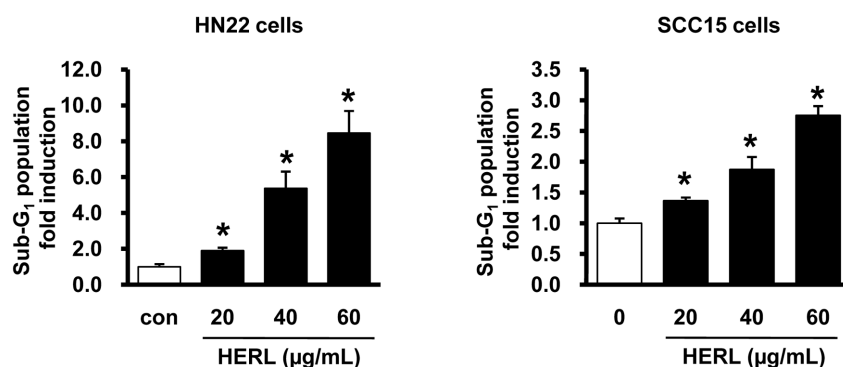

Figure 2. The effect of hexane extract of *Rheum undulatum* L. (HERL) on cell accumulation in sub-G$_1$ phase. The sub-G$_1$ population was measured by PI staining and flow cytometry analysis. The bar graph represents three independent experiments and mean±SD. *P<0.05 compared to control group.

to the control (Figure 3). We next determined the level of PARP cleavage by Western blot analysis (Figure 4). After HERL treatment for 48 h, cleaved PARP was detected in the HN22 and SCC15 cells. Thus, we clearly showed that HERL induced apoptosis in HN22 and SCC15 cells to inhibit cell growth.

HERL inhibited anti-apoptotic protein Sp1 and survivin in oral cancer cell lines

In previous studies, it was reported that targeting of Sp1 protein is a good treatment strategy for various cancer cells (Lou *et al*, 2005; Safe and Abdelrahim, 2005; Abdelrahim
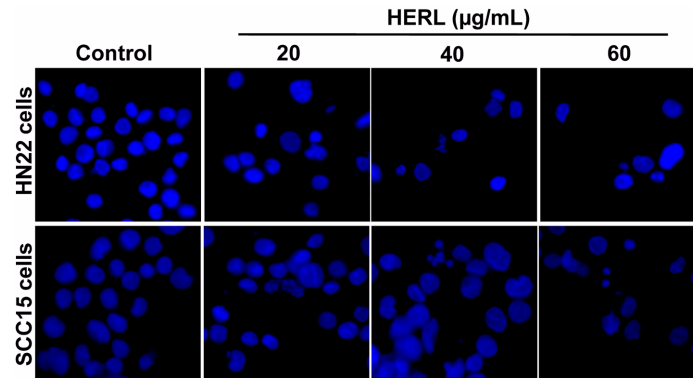
Figure 3. The effect of hexane extract of *Rheum undulatum* L. (HERL) on the condensation and fragmentation of nucleus in HN22 and SCC15 cells. Fluorescence microscopy images of DAPI-stained HN22 and SCC15 cells showing the appearance of apoptotic morphology.
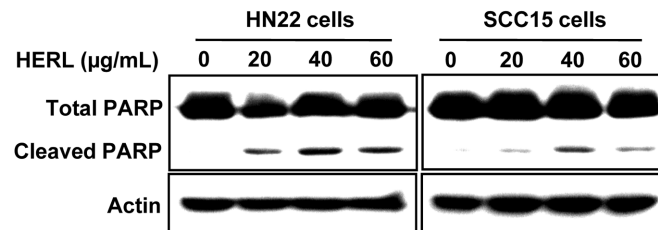

Figure 4. The effect of hexane extract of *Rheum undulatum* L. (HERL) on the cleavage of poly(ADP-ribose) polymerase (PARP) in HN22 and SCC15 cells. HN22 and SCC15 cells were treated with 20, 40 or 60 μg/mL of HERL for 48 h, and then the protein levels of cleaved PARP were determined by Western blot analysis. Actin was used to normalize the protein loading from each treatment.
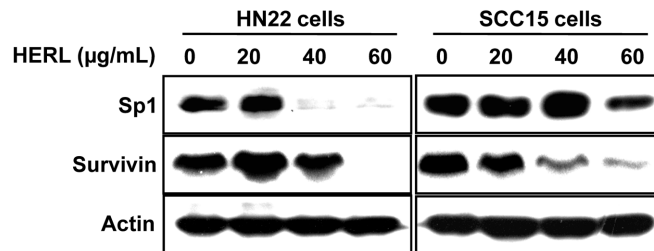

Figure 5. The effect of hexane extract of *Rheum undulatum* L. (HERL) on the regulation of Sp1 and survivin protein expression. Sp1 and survivin protein levels were analyzed by Western blot analysis.

et al, 2006; Papineni *et al*, 2009; Lu and Archer, 2010) and Sp1 protein regulates survivin protein as the downstream protein to induce apoptosis in cancer cells (Shin *et al*, 2010). Therefore, we assessed whether HERL affects Sp1 and survivin protein to induce apoptotic cell death in HN22 and SCC15 cells. As shown in Figure 5, HERL-treated HN22 and SCC15 cells exhibited decreased Sp1 and survivin expression in a dose-dependent manner. These results suggest that HERL might inhibit Sp1 and survivin protein to exert its apoptotic activity in HN22 and SCC15 cells.

Discussion

Natural extracts from edible plants have been identified

as excellent candidates for cancer therapeutics based on safety and efficacy (Stewart *et al*, 2003). Recently, *Rheum undulatum* L. was screened for anti-cancer activity *in vitro* using human breast, ovary, cervix and lung cancer cell lines (Kang *et al*, 2008). In that study, emodin showed strong cytotoxicity in both estrogen receptor (ER)-positive and -negative breast cancer cell lines (Kang *et al*, 2008). In addition, HERL was shown to inhibit angiogenic activity in a zebrafish model (He *et al*, 2009). These studies suggest that HERL has potential benefits for the treatment of various cancers. However, the anticancer activity of HERL against oral cancer cells has not been well established. Therefore, we focused on the anticancer activity of HERL in human oral cancer cells. Our first purpose was to examine the anti-proliferative and pro-apoptotic effects

of HERL on oral cancer cell lines and the other was to determine its molecular mechanism in HERL-induced apoptosis. First, we found that HERL decreased the number of viable cells in a dose-dependent manner, showing that HERL has an inhibitory effect on cell growth in HN22 and SCC15 cells. We found that HERL induced the apoptosis of HN22 and SCC15 cells as evidenced by the accumulation of sub-G$_1$ phase cells, distinct chromatin condensation and nuclear fragmentation of the nucleus and the cleavage of PARP protein, a typical apoptotic marker.

Sp1 is a member of the mammalian transcription factor family that binds to GC-rich sites containing GC-boxes. Recently, it was reported that Sp1 protein is over-expressed in many human tumors and cancer cell lines (Zannetti *et al*, 2000; Chiefari *et al*, 2002; Wang *et al*, 2003; Hosoi *et al*, 2004; Yao *et al*, 2004). Sp1 has also been implicated in multiple cell processes, including apoptosis, through activation of the FAS-ligand (Kavurma *et al*, 2001; Sun *et al*, 2001). In addition, survivin, an inhibitor of apoptosis and a key regulator of mitosis, is up-regulated in a variety of cancer cells and is often associated with a worse prognosis (Sun *et al*, 2001). Interestingly, several studies have shown that survivin expression can be regulated by Sp1 protein because it contains highly GC-rich sequences within its promoters (Li and Altieri, 1999; Wu *et al*, 2005; Li *et al*, 2006; Shim *et al*, 2010). Therefore, we examined the effects of HERL on the expression of Sp1 and its downstream target, survivin, as the key molecular factors in human oral cancer cells. Our results show that HERL decreases the expression levels of Sp1 and survivin proteins, suggesting that HERL inhibits the growth and induces apoptosis in HN22 and SCC15 cells through the regulation of Sp1 and survivin.

In summary, our data showed HERL exerts pro-apoptotic and anti-proliferative effects in HN22 and SCC15 human oral cancer cells and that Sp1 and survivin play important signaling roles in HERL-induced apoptosis. Therefore, we suggest that HERL is a promising anti-cancer drug candidate for the effective treatment of oral cancer.

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