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Acetylshikonin inhibits growth of oral squamous cell carcinoma by inducing apoptosis

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ABSTRACT

Objectives: Recently, shikonin derivatives from *Lithospermum erythrorhizon* have been suggested as potential chemotherapeutic agents against numerous types of cancers in addition to their traditional uses, e.g., as anti-inflammatory agents. Acetylshikonin, one of shikonin derivatives, has also been reported to possess anticancer activity. However, few studies of the effectiveness of acetylshikonin against cancer cells have been conducted, and there are no studies of oral cancers. In this study, we investigated the usefulness of acetylshikonin as a treatment regimen for oral cancers by observing the growth inhibitory function of acetylshikonin and the involved mechanisms.
Designs: The viability, cell cycle, and ratio of apoptotic cells of oral squamous cell carcinoma (OSCC) cells were observed after treatment with acetylshikonin using MTT assay, flow cytometric analysis, and Annexin V/PI staining, respectively. In addition, molecular changes of apoptosis-related pathways and the role of reactive oxygen species (ROS) were analyzed in acetylshikonin-treated cells.
Results: We observed that acetylshikonin significantly suppressed the growth of OSCC cells by inducing apoptotic cell death, and acetylshikonin affected the viability of a normal keratinocyte cell line HaCaT to a lesser degree, suggesting that acetylshikonin may be a good chemotherapeutic reagent with less toxicity to normal tissues. In addition, we found that acetylshikonin-induced apoptosis of OSCC cells is mediated by ROS as well as G2 cell cycle arrest. ROS production in response to acetylshikonin treatment enhanced the phosphorylation of JNK and p38 MAPK, which are in the major pathways of apoptotic cell death mechanisms.
Conclusions: In summary, our data suggest that acetylshikonin is a strong candidate for use as a selective chemotherapeutic agent for the treatment of OSCC.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms in the oral cavity (Greenlee, Hill-Harmon, Murray, & Thun, 2001), and 5-year survival rates over the past two decades have remained constant at 60% (Jemal et al., 2006). In addition to no improvement in the survival rate, adverse effects resulting from treatment have been a problem. For example, a surgical intervention can often result in facial deformities and functional disturbances in the oro-maxillofacial region. High-dose radiotherapy usually evokes significant complications, such as mucositis, skin reactions and dysphagia. To improve these side effects, numerous drugs capable of treating cancers or decreasing tumor volume have been tried, and a number of natural derivatives have been investigated as chemotherapeutic agents, which are accompanied by minimal adverse effects (Nam et al., 2007; Rahman, Amin, & Shin, 2010). Shikonin and its derivatives are isolated from the roots of *Lithospermum erythrorhizon*, which is a medicinal plant. This plant has traditionally been utilized for the treatment of macular eruptions, measles, sore-throats, carbuncles and burns for thousands of years in Asian countries (Chen, Yang, Oppenheim, & Howard, 2002). Shikonin has also been evaluated as a chemotherapeutic agent against various tumors including colon adenocarcinoma, epidermoid carcinoma, leukemia, and prostate cancer (Gaddipati et al., 2000; Kwak et al., 2014; Singh, Gao,

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Lebwohl, & Wei, 2003; Yoon, Kim, Lim, Jeon, & Sung, 1999) and was shown to possess anti-cancer properties as well as traditionally known anti-inflammatory and antibacterial activities (Wu et al., 2004a; Wu et al., 2004b). However, shikonin has limited potential as an anticancer drug due to its toxicity. To find more effective antitumor drug candidate with fewer side effects, the biologic activities and toxicities of shikonin derivatives have been tested. Acetylshikonin, one of the shikonin derivatives, is effective in healing infected wounds and has been found to have less toxicity than shikonin and also to possess anticancer activity (Xuan & Hu, 2009; Zeng, Liu, & Zhou, 2009). Although these previous studies have observed the anticancer effects of shikonin and acetylshikonin on a few types of cancers, few studies have been conducted on oral cancers (Min et al., 2008). In particular, no study has investigated the utilization of acetylshikonin as a chemotherapeutic agent against oral cancers. Thus, in this study, we aimed to address the possible usefulness of acetylshikonin on oral cancers, especially on OSCC cells. In addition, we focused on whether acetylshikonin can induce cell death in OSCC and on the molecular mechanism of acetylshikonin initiated cell death. The present study observed that acetylshikonin can suppress the growth of OSCC cells by inducing apoptotic cell death, and found that reactive oxygen species (ROS) formation is one of the major inducers of apoptosis.

2. Materials and methods

2.1. Maintenance of cell culture

The oral squamous cell carcinoma (OSCC) cell line, Ca9-22, and human keratinocyte cell line, HaCaT, were purchased from JCRB (JCRB, Osaka, Japan) and the from American Type culture Collection (ATCC; Manassas, VA, USA), respectively. The Ca9-22 and HaCaT cell lines were maintained in Eagle's minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (V/V) heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (GIBCO-BRL, Rockville, MD, USA), respectively. All cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Reagents and chemicals

Acetylshikonin was kindly provided by Prof. Young Whan Choi of Miryang Campus, Pusan National University. Acetylshikonin was dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 4 mM, stored in a dark colored bottle at −20 °C, and diluted to the indicated concentration in MEM or DMEM before use. The primary antibodies used included the following: rabbit antibodies against phospho-SAPK/JNK (Thr183/Thr185), SAPK/JNK, phospho-p38 MAP Kinase (Thr180/Tyr182), p38 MAP kinase, cleaved caspase-3, cleaved PARP (Asp214) and phospho-ERK1/2 antibodies (Cell Signaling Technology Inc., Beverly, MA, USA); mouse antibodies against PARP, ERK1/2 (Cell Signaling Technology Inc.) and actin (Santa Cruz Biotechnology, CA, USA). All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

2.3. Cell viability assay

Cell viability of Ca9-22 and HaCaT cells was measured by MTT assay. Cells were plated in 96-well plates (1×10^4/well), incubated overnight to reach approximately 80% confluence, and then treated with varying concentrations of acetylshikonin (0–2 μM) for 24 or 48 h. Cells grown in a medium containing an equivalent amount of DMSO without acetylshikonin served as a control. At a given interval, media were removed, and 100 μl of MTT (5 mg/ml) was added to each well. The plates were then further incubated for 4 h at 37 °C. The resulting formazan crystals were solubilized in 200 μl of DMSO. The absorbance at 570 nm of colored solutions was quantified using a PerkinElmer Victor-3 spectrophotometer. Cell viability was measured as the percent ratio of absorbance in acetylshikonin-treated cells over the control. Each condition tested in triplicates in a single trial, and data were obtained from at least 3 independent experiments.

2.4. Cell cycle analysis

Cells were harvested by centrifugation at 1000 rpm for 5 min, fixed in 70% ethanol and washed with cold PBS. After suspended in 1 ml PBS containing RNase A (10 μg/ml) cells were incubated at 4 °Cfor 30 min and analyzed with a flow cytometry system (FACS, Beckman Coulter, USA).

2.5. Annexin V-FITC/PI staining assay

Apoptotic cells were evaluated by flow cytometry and fluorescence microscopy. Cells were treated with 1 μM acetylshikonin for 24 h. For flow cytometry, 1×10^6 cells in 500 μl of binding buffer were stained with Annexin V-FITC (10 μl) and propidium iodide (10 μl) for 15 min. Cells were analyzed with a flow cytometry system (Beckman Coulter), and data were analyzed for cell-cycle parameters and apoptosis using Multicycle software.

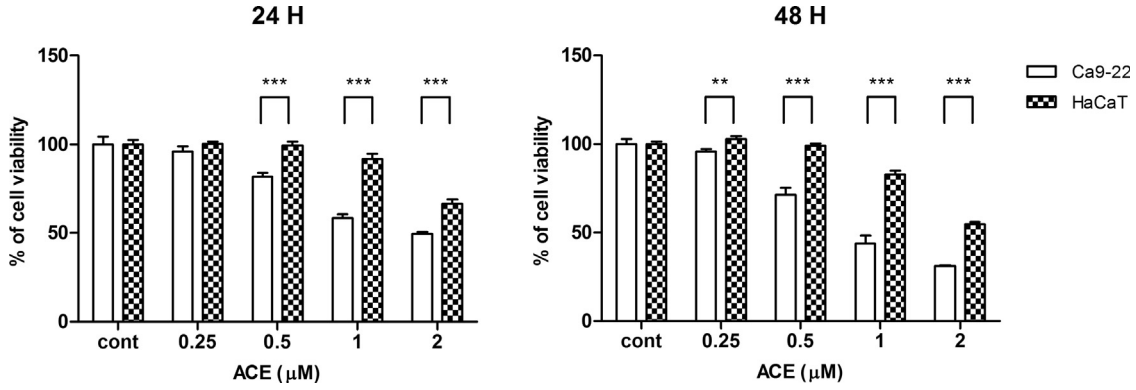

Fig. 1. Acetylshikonin effectively induced cell death in Ca9-22 OSCC cells, but normal keratinocyte HaCaT cells were much less sensitive to acetylshikonin treatment. Cells were grown in 96-well plates and treated with 0, 0.25, 0.5, 1 or 2 μM of acetylshikonin for 24 h or 48 h. DMSO was used as a vehicle control. Cell viability was measured using the MTT assay. Bars represent means ± SEM for three independent experiments (**, $p < 0.01$; ***, $p < 0.001$).