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Mechanism of arctigenin-mediated specific cytotoxicity against human lung adenocarcinoma cell lines

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ABSTRACT

The lignan arctigenin (ARG) from the herb *Arctium lappa* L. possesses anti-cancer activity, however the mechanism of action of ARG has been found to vary among tissues and types of cancer cells. The current study aims to gain insight into the ARG mediated mechanism of action involved in inhibiting proliferation and inducing apoptosis in lung adenocarcinoma cells. This study also delineates the cancer cell specificity of ARG by comparison with its effects on various normal cell lines. ARG selectively arrested the proliferation of cancer cells at the G_0/G_1 phase through the down-regulation of NPAT protein expression. This down-regulation occurred via the suppression of either cyclin E/CDK2 or cyclin H/CDK7, while apoptosis was induced through the modulation of the Akt-1-related signaling pathway. Furthermore, a GSH synthase inhibitor specifically enhanced the cytotoxicity of ARG against cancer cells, suggesting that the intracellular GSH content was another factor influencing the susceptibility of cancer cells to ARG. These findings suggest that specific cytotoxicity of ARG against lung cancer cells was explained by its selective modulation of the expression of NPAT, which is involved in histone biosynthesis. The cytotoxicity of ARG appeared to be dependent on the intracellular GSH level.

Introduction

Lung cancer is a major worldwide health problem and has accounted for approximately 16% of global cancer deaths (Pisani et al., 1999). There are 2 main types of lung cancer: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). Lung adenocarcinoma, which belongs to the NSCLC group, constitutes approximately 75–85% of all lung cancers (Greenlee et al., 2001).

Chemotherapy has been established as a standard treatment for NSCLC; however, despite the success of chemotherapy, it has a limited ability to improve a patient's symptoms and quality of life (Ten Bokkel Huinink et al., 1999). While some lung cancer therapies have shown promising results for the control of disease progression, the toxicity of this therapeutics often restricts the completion of the recommended dose. Therefore, the identification of an anti-cancer agent with high selectivity against NSCLC merits further investigation and is preferable than searching for agents with high toxicity.

Increasing attention has been paid to the pharmaceutical value and the biological activity of herbal plant medicines over the last decade. Numerous bioactive compounds isolated from herbal plants exhibit selective toxicity toward tumorigenic tissues, and thus display cancer-targeting properties (Quasney et al., 2001). The lignans compose one group of chemical compounds found in plants, and include arctigenin (ARG), a dibenzylbutyrolactone lignan isolated from *Arctium lappa* L. ARG has been reported to possess many important pharmacological or biological properties, including anti-oxidative, anti-tumorigenic, and anti-inflammatory activities (Awale et al., 2006; Matsumoto et al., 2006). Our previous study showed that ARG specifically inhibited the proliferation of lung adenocarcinoma (A549) cells, which might consequently lead to the induction of apoptosis (Susanti et al., 2012). Although the anti-cancer activity of ARG appears to be promising, the mechanism of its action is not yet fully understood. Several studies have reported that ARG blocks the unfolded protein response and activates Akt in glucose-deprived solid tumors (Kim et al., 2010; Awale et al., 2006). However, the mechanism proposed for the glucose-deprived cancer cells may not necessarily be applicable to our current study. It is known that the inhibition of cell proliferation is involved in the anti-cancer mechanism of ARG; however, the signal transduction pathways leading to cell cycle arrest are entirely unknown. ARG has been shown to induce cell cycle arrest at the G_0/G_1 phase in gastric cancer cells by modulation of regulatory proteins such as Rb and

Abbreviations: ARG, arctigenin; GSH, glutathione; G_0/G_1, Gap 0/Gap 1; NPAT, nuclear protein of the ataxia telangiectasia locus; CDK2, cyclin-dependent kinase 2; CDK7, cyclin-dependent kinase 7; akt-1, alpha serine/threonine-protein kinase.

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cyclin D1 (Jeong et al., 2011), and at the G_2/M phase in colorectal cancer cells via the regulation of the Wnt/β-catenin signaling pathway (Yoo et al., 2010). Furthermore, ARG was detoxified by glutathione (GSH) in liver cells, indicating that the expression level of GSH synthase may be an additional factor controlling cell susceptibility to this agent (Moritani et al., 1996). Therefore, the mechanism of action of ARG is likely to vary among tissues and cancer cell types.

The current study aims to gain insight into the mechanisms involved in ARG-mediated inhibition of proliferation and induction of apoptosis in lung adenocarcinoma. Furthermore, this study aims to delineate the specificity of ARG via comparative studies on various normal cell lines. The results of this investigation will provide valuable new information on the usefulness of ARG as a promising chemotherapeutic agent with few adverse effects.

Materials and methods

Isolation and purification of ARG

The isolation and purification of ARG from *A. lappa* L. extract were performed as described previously in Susanti et al. (2012).

Cell cultures

Various human normal embryo fibroblast (OUMS-36, OUMS-36T-2F, and OUMS-36T-5F) and lung adenocarcinoma (A549) cell lines were purchased from the Japanese Cancer Research Resources Bank (JCRB, Ibaraki, Japan). Cells were cultured in DMEM supplemented with 10% FBS (Fetal Bovine Serum) in a humidified 5% CO_2 atmosphere at 37 °C.

Cell viability assay

Cells suspended in DMEM were seeded at 1×10^4 cells (100 μl) per well in 96-well plates and pre-incubated overnight in a humidified atmosphere of 5% CO_2 at 37 °C. After a 24-h incubation with varying concentrations of ARG, cell viability was determined using MTS assay kits according to the manufacturer's instructions (Cell Titer 96® AQueous non-radioactive cell proliferation assay, Promega Co., Madison, USA). All experiments were performed in triplicate, and cell viability was expressed as the relative viability of the treated cells compared to untreated cells (control).

Cell cycle analysis

Cells (3×10^5) were seeded in 60-mm culture dishes and pre-incubated for 24 h at 37 °C. Cells were washed with PBS before the replacement of the medium, and then cultured in DMEM supplemented with or without ARG (6 μg/ml). After a 24 h incubation, cells were harvested using ICT Accutase™ cell detachment solution (Innovative Cell Technologies, Inc, San Diego, CA, USA), washed with PBS, fixed with 70% cold ethanol, and incubated overnight at 4 °C. Ethanol was removed by decantation, and cells were re-suspended in PBS on ice for 10 min. Cell suspensions were centrifuged at 1000 rpm for 10 min, re-suspended in a 250 U/ml RNase solution, and incubated for 20 min at room temperature. After 200 μg/ml propidium iodide solutions was added, the cell suspensions were transferred and filtered through a 35 mM nylon filter into flow cytometer tubes. Data acquisition and analysis were performed by a FACS Calibur flow cytometer system (BD Biosciences).

Western blot analysis

Proteins were isolated using PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Korea) from the lysate of 2.5×10^6 cells that had been treated with 6 μg/ml ARG for 24 h. The protein concentration was measured using Quant-iT™ Protein Assay Kits (Invitrogen, USA) according to the manufacturer's instructions. Protein samples were dissolved in an equal amount of sample buffer solution (EzApply, Atta, Osaka, Japan). Proteins (10 μg) were separated on 12.5% SDS–PAGE gels (e-PAGEL, E-R12.5L, ATTO, Tokyo, Japan) run at 15–20 mA in running buffer (EzRun, ATTO, Tokyo, Japan). The proteins were transferred to PVDF membranes by using the iBlot™ Dry Blotting System (Invitrogen, Tokyo, Japan). The PVDF membranes were treated with blocking buffer (Blocking One, Nacalai Tesque, Kyoto, Japan) for 1 h and washed with TBS (1× PBS in 0.1% Tween-20). Subsequently, the membranes were incubated with primary antibody (β-actin, CDK2, p-CDK, CDK7, Cyclin E, Cyclin H, Rb, P-Rb, and NPAT) with gentle shaking overnight. After washing, the membranes were incubated with an HRP-linked anti-rabbit IgG secondary antibody for 1 h and then washed again. The detection of target proteins was performed using a luminescent image analyzer (ImageQuant LAS 4000 mini, GE Healthcare, Uppsala, Sweden) and Amersham ECL Advance Western Blotting Detection Kits (GE Healthcare, Buckinghamshire, UK). The relative intensities (%) of protein bands were measured using Image J software version 1.47d.

Total RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from either untreated or treated cells (1×10^6) by using the AquaPure RNA Isolation Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The quality of the isolated RNA was checked using MultiNA Reagent Kits on a microchip electrophoresis system (MultiNA-Biotech, Shimadzu, Japan) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed to produce cDNA by using High Capacity RNA-to-cDNA Kits according to the manufacturer's instructions (Applied Biosystems, USA). cDNA samples (20 μl aliquots) were stored at −20 °C. For real-time PCR analysis, the cDNAs were amplified in a StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA) by using Fast SYBR Green Master Mix (Applied Biosystems, CA, USA), according to the manufacturer's instructions. Table 1 lists the sequences of the primers that were used for quantitative real-time RT-PCR analysis. All analyses were performed in triplicate, and the gene expression levels were normalized to the housekeeping gene ACTB. Fold changes in gene expression were calculated on the basis of the standard curve, which was constructed using the calibration data produced by the StepOne software.

Effect of GSH depletion on ARG cytotoxicity

The effect of GSH depletion on the cytotoxicity of ARG was studied using the GSH synthase inhibitor L-buthionine-(S,R)-sulfoximine (BSO). Cells suspended in DMEM were seeded at 1×10^4 cells (100 μl) per well in 96-well plates and pre-incubated in a humidified 5% CO_2 atmosphere at 37 °C for 10 h. The cells were incubated with 50 μM of BSO for 14 h, and subsequently treated with varying concentrations of ARG for 24 h. Cell viability was then determined using MTS assay kits according to the manufacturer's instructions (Cell Titer 96® AQueous non-radioactive cell proliferation assay, Promega Co., Madison, USA). All experiments were performed in triplicate, and the cell cytotoxicity was expressed as the relative viability of treated cells compared to that of untreated control cells. In the case of BSO pretreatment, control cells were treated with BSO only. The ED_{50} of ARG with or without BSO pretreatment was estimated by fitting the following formula to the titration curve:

$$y = \frac{\beta_3 + \beta_4}{\{1 + \exp(\beta_1 + \beta_2 x)\}}$$