Chemotherapeutic Effect of Withaferin A in Human Oral Cancer Cells

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Received 17 July 2015; accepted 7 August 2015; published 10 August 2015

Abstract

Withaferin A (WA) is a bioactive compound derived from a medicinal plant *Withania somnifera* and has potential therapeutic effects against various types of cancers. The purpose of this study is to investigate an apoptotic effect of WA and identify its molecular target in HSC-3 and HSC-4 human oral cancer cell lines using Trypan blue exclusion assay, DAPI staining and western blotting. WA inhibited cell viability and induced apoptosis in a concentration- or time-dependent manner, as evidenced by induction of nuclear condensation and fragmentation, activation of caspase 3 and poly (ADP-ribose) polymerase (PARP) cleavage. WA-induced apoptosis was partly diminished by Z-VAD, a pancaspase inhibitor. WA also increased Bim and Bax protein in HSC-3 and HSC-4 cells, respectively. These results suggest that WA may be a potential chemotherapeutic drug candidate against human oral cancer.

Keywords

Oral Cancer, Withaferin A, Apoptosis, Bim, Bax

1. Introduction

Anticancer agents including cisplatin and pingyangmycin have been generally used in head and neck and tongue cancer treatment by suppressing tumor size and inhibiting metastasis [1] [2]. However, it has been reported to have toxicity, side effect and resistance to apoptosis in ovarian and tongue cancer [2] [3]. The failure of current therapies in adrenocortical carcinoma has been reported to be correlated with cytotoxic drugs containing cisplatin, etoposide, mitotane and doxorubicin [4]. For this reason, novel agents with low toxicity are necessary to be

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developed for the treatment of various cancers including oral cancer.

Natural compounds derived from plant sources have steadily been known as invaluable source of therapeutic agents [5]. They were also used as traditional medicines for the treatment of various cancers because of their potential anti-cancer effects [6]-[8]. Withania somnifera is a plant with bioactive compounds, which has been known as winter cherry or Indian ginseng [9]. WA, derived from Withania somnifera, has therapeutic effects, such as anti-inflammatory, anti-angiogenesis and anti-cancer effects in various cancers [9]-[11]. It has been reported that WA suppressed cell growth and induced apoptosis in breast cancer cells in vitro and in vivo [12]. WA also induced apoptosis in human melanoma cells [13]. Thus, WA has a great possibility to become an effective cancer therapy.

Bcl-2 family proteins have been characterized as cell survival factors (Bcl-2, Bcl-XL, Mcl-1 and so on) and pro-apoptotic factors (Bak, Bax, Bim, Bid and so on), which can regulate mitochondria-dependent apoptosis [14]. These proteins include at least one of four BCL-2 homology domains, BH1 to BH4 [15]. BH3-only proteins, Bim and Bid act upstream of Bax and Bak with BH1-BH3 domains to induce apoptosis [16]. It has been demonstrated that Bim activates the apoptotic proteins Bax and Bak, leading to cytosolic release of cytochrome c from mitochondria and induce apoptosis [17]. Recently, WA-induced apoptosis has been studied in various cancer cell lines, such as breast [18], prostate [19], pancreatic [20], ovarian [21], lung [22], head and neck cancer cell lines [23]. However, the detailed molecular target behind the apoptosis of human oral cancer cells is not clear yet. Therefore, it is valuable to investigate the molecular target of WA-induced apoptosis in human oral cancer cells. In this study, our group investigated the efficacy of WA through the regulation of Bcl-2 family proteins in human oral cancer cells.

2. Materials and Methods

2.1. Cell Culture and Chemical Treatment

HSC-3 and HSC-4 cells were provided by Hokkaido University (Hokkaido, Japan). Cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO2 incubator. All experiments were prepared in cells cultured at 50% - 60% confluence. Withaferin A (Sigma, St. Louis, Mo, USA) was dissolved in 0.1% DMSO (vehicle control) and stored at −20°C. Z-VAD (Minneapolis, Minnesota, USA) was treated as a pan-caspase inhibitor into cell lines.

2.2. Trypan Blue Exclusion Assay

The growth inhibitory effect of WA was determined with trypan blue solution (Gibco, Paisley, UK). Cells were stained with trypan blue (0.4%), and then viable cells were counted using a hemocytometer.

2.3. 4′-6-Diamidino-2-Phenylindole (DAPI) Staining

To detect nuclear morphological changes of apoptotic cells, cells were stained with DAPI solution (Sigma-Aldrich, Louis, MO, USA). Briefly, cells were fixed in 100% methanol at RT for 10 min, deposited on slides, and stained with DAPI solution (2 μg/ml). The morphological changes of apoptotic cells were observed under a fluorescence microscopy.

2.4. Western Blot Analysis

Whole-cell lysates were prepared with lysis buffer and protein concentration in each sample was measured using a DC Protein Assay Kit (BIO-RAD Laboratories, Madison, WI, USA). After normalization, equal amounts of protein were separated by SDS-PAGE and then transferred to Immun-Blot™ PVDF membranes. The membranes were blocked with 5% skim milk in TBST at RT for 2 hr, and incubated with primary antibodies and corresponding HRP-conjugated secondary antibodies. Antibodies against cleaved PARP, cleaved caspse-3, Bax and Bim were purchased from Cell Signaling Technology, Inc., (Charlottesville, VA, USA). Actin antibody was obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). The immunoreactive bands were visualized by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.5. Statistical Analysis

Student’s t-test was used to determine the significance of differences between the control and treatment groups;
values of $p < 0.05$ were considered significant.

3. Results

3.1. WA Reduces the Viability in HSC-3 and HSC-4 Cells

To explore the potential anti-cancer effects of WA in human oral cancer cells, we examined the effects of WA by cell counting after the treatment with DMSO or various concentrations for 24 hr as well as certain concentration (1 μM for HSC-3 cells and 0.8 μM for HSC-4 cells) for each indicated time points (0, 3, 6, 12 and 24 hr). As shown in Figure 1, the viability of HSC-3 and HSC-4 cells was notably decreased in a concentration- and time-dependent manner. These results indicate that WA can decrease cell viability in human oral cancer cells.

3.2. WA Increased Apoptosis in HSC-3 and HSC-4 Cells

To determine whether the growth inhibitory effect of WA was associated with apoptosis, we performed using DAPI staining. As presented in Figure 2, the exposure of cells to WA exhibited a noticeable increase in the distinct features of apoptotic cells such as chromatin condensation and nuclear fragmentation. These results suggest that growth inhibitory effect of WA may be associated with induction of apoptosis.

3.3. WA-Induced Apoptosis Is Associated with Activation of Caspase 3 in HSC-3 and HSC-4 Cells

Next, we carried out western blot analysis using antibody against cleaved PARP and caspase 3. The results showed that the augmentation of cleaved PARP and caspase 3 by WA was in a concentration-dependent in HSC-3 and HSC-4 cells (Figure 3(a)). Also, the results from western blot analysis showed that exposure of cells to WA caused a markedly induction of cleaved PARP in a time-dependent manner (Figure 3(b)). To identify the involvement of caspase 3 activation in WA-mediated apoptotic cell death, we used a pancaspase inhibitor, Z-VAD and the apoptosis induced by WA was modestly abolished by Z-VAD (Figure 3(c)). These results suggest that WA has caspase-dependent apoptotic activity in HSC-3 and HSC-4 cells.

![Figure 1](image-url) Withaferin A inhibits cell viability in human oral cancer cells. HSC-3 and HSC-4 cells were treated with DMSO or multiple concentrations of WA for 24 hr. A. The effect of WA on cell viability was examined using Trypan blue exclusion assay. B, Each cell lines were harvested at different time points (0, 3, 6, 12 and 24 hr). The graphs were expressed the mean ± S.D. of triplicate experiments and significance ($p < 0.05$) compared with the DMSO-treated group was indicated (*).
Figure 2. WA increases nuclear fragmentation and condensation in human oral cancer cells. HSC-3 and HSC-4 cells were treated with DMSO or various concentrations and time-dependent of WA for 24 hr and/or each time point. A and B, Nuclear condensation and DNA fragmentation were stained with DAPI solution as mentioned in Material and Methods (×400). DAPI-stained cells were observed by fluorescence microscopy.

Figure 3. WA induces apoptosis in HSC-3 and HSC-4 cells. Total proteins were prepared for western blot with indicated antibodies. (a) HSC-3 and HSC-4 cells were treated with multiple concentration of WA; (b) Effect of WA was confirmed time dependently; (c) Pan-caspase inhibitor (Z-VAD) was used to evaluate the involvement of caspase 3 for WA-induced apoptosis, and actin was used as a loading control.
3.4. WA Induces Apoptosis through Regulation of Bim and Bax

To clarify whether the apoptotic effect of WA is related to the regulation of Bcl-2 family proteins, we examined protein levels of Bim and Bax. As shown in Figure 4, WA increased Bim expression in HSC-3 cells and caused an increase in Bax in HSC-4 cells. These results suggest that WA-induced apoptosis may be associated with the regulation of several Bcl-2 family proteins in a cell line-specific manner.

4. Discussion

The natural synthetic or biological compounds have been used to treat cancers. Numerous studies have been demonstrated that natural compounds play critical roles in the induction of apoptosis in various cancers such as gastric, breast, lung and others cancers [24]-[26]. For example, a cacalol derived in Asian herbal plant has exerted anti-proliferative and apoptotic effect in breast cancer cells [27]. Eugenol has reported to induce apoptosis in human HT 29 colon cancer cells [28]. Previously, our group has demonstrated that several natural compounds such as *Codonopsis lanceolata* and *Tricholoma matsutake* extracts exerted apoptotic activities through the augmentation of pro-apoptotic proteins, Bid and Bax levels in oral cancer [29]. Herein the present study, we showed that a natural compound, WA reduces cell viability and induced apoptosis in human oral cancer cells (Figures 1-3) indicating that WA may have anti-carcinogenic activities against oral cancer. WA has been reported as promising anti-cancer drug candidate due to its cytotoxic and apoptotic properties [30]. WA is known having a critical role in the inhibition of abnormal cell proliferation occurring in oral carcinogenesis [31]. Furthermore, anti-cancer effect of WA has demonstrated that it reduced cell viability and proliferation in adrenocortical carcinoma [32]. Thus, our results proved that WA has growth inhibitory and apoptotic effect in oral cancer cells.

The apoptotic pathway includes the extrinsic (cytoplasmic) and intrinsic (mitochondrial) pathway [33]. Permeabilization of the mitochondrial outer membrane is importantly associated with Bcl-2 family proteins that regulate the integrity of the mitochondria [34]. In particular, natural compound regulated the Bim expression of Bcl-2 member and induced apoptosis by cytochrome C release [35]. *Antrodia camphorate* extracts have been demonstrated that activation of caspase-3, -8, and -9 and the increase in the cytosolic level of cytochrome c were accompanied by increasing the expression levels of Bak, Bad and Bim in HeLa and C-33A cells [36]. Rhein also increased the expression of Bim and FOXO3a in MCF-7 and HepG2 cells during apoptosis [37]. Our group also has demonstrated that an analogue of curcumin, dibenzylideneacetone enhanced Bax expression resulting in apoptosis in oral cancer cells [38]. In the light of the potential effect by natural compounds on Bcl-2 family proteins, we assumed that WA could have pro-apoptotic properties through regulation of Bcl-2 family proteins. In
practice, WA-induced apoptosis in human melanoma cells has reported that it correlated with mitochondrial pathway, which is regulated by Bcl-2 family protein, Bax and Bak and caspase-dependent pathway [13]. In this study, we also identified whether WA-induced apoptosis affects Bcl-2 family proteins and the results demonstrated that Bim and Bax were affected by WA in HSC-3 and HSC-4 cells, respectively (Figure 4). These results suggest that the up-regulation of Bim and Bax may be required for WA-induced apoptosis in oral cancer cells. Therefore, it would be valuable to further investigate continuously in future study.

5. Conclusion
In conclusion, our results show that WA reduces cell viability and upregulates the expression of Bim and Bax, leading to apoptosis in HSC-3 and HSC-4 oral cancer cells. Thus, these provide the basis that WA has an attractive chemotherapeutic drug candidate for therapy of oral cancer, although anti-tumorigenic effect of WA in vivo model is needed.

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