Journal of Ethnopharmacology 153 (2014) 250–257

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Anti-melanoma activity of *Cynanchi atrati* Radix is mediated by regulation of NF-kappa B activity and pro-apoptotic proteins

Seung-Wan Son [a], Hyeong-Geug Kim [b], Jong-Min Han [b], Jin-Seok Lee [b], Min-Kyung Choi [b], Jong-Seok Lee [c], Chang-Gue Son [b,*]

[a] *Department of Biomedical Engineering, College of Health Science, Korea University, Seongbuk-Gu, Seoul 136-703, Republic of Korea*
[b] *Liver and Immunology Research Center, Daejeon Oriental Hospital of Oriental Medical College of Daejeon University, 22-5 Daehung-dong, Jung-gu, Daejeon 301-724, Republic of Korea*
[c] *Gyeonggi Biocenter, Gyeonggi Institute of Science & Technology Promotion (GSTEP), Suwon, Gyeonggi-do 443-270, Republic of Korea*

ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form
16 February 2014
Accepted 17 February 2014
Available online 26 February 2014

Keywords:
Cynanchi Atrati Radix
Melanoma
B16F10
Apoptosis
Nuclear factor kappa B

ABSTRACT

Ethnopharmacological relevance: *Cynanchi atrati* Radix has been traditionally prescribed for patients with inflammatory fever or chronic tumoral disorders. Melanoma is one of the most devastating cancer types, in which overexpression of nuclear factor kappa B (NF-κB) enables the cancer to survive without apoptosis. To identify a potential anti-melanoma candidate, we evaluated the apoptotic activity of an ethanol extract of *Cynanchi atrati* Radix (CAE) on melanoma and its underlying mechanisms.

Materials and methods: Sixty C57BL/6 N mice with melanoma were orally administrated CAE (100 or 200 mg/kg) or distilled water for 10 days. Survival, tumor weight and volume were monitored and measured. Intratumoral apoptotic change was measured using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. To confirm the pro-apoptotic activity of CAE (10, 50 or 100 μg/mL) compared to positive drug (10 μg/mL of IKK-2 inhibitor IV), cell proliferation, caspase-3/7 activity, flow cytometric analysis, TUNEL and DAPI staining, immunoblotting and gene expression analyses for apoptosis-associated genes were conducted using B16F10 cell line.

Results: CAE administration remarkably improved survivability with a significant reduction in tumor weight ($p < 0.01$) and volume ($p < 0.01$), as well as increased apoptotic bodies in melanoma tissue. The CAE treatment significantly inhibited proliferation of B16F10 cells ($p < 0.001$), but increased caspase-3/7 activity ($p < 0.01$ or 0.001) and apoptotic population. The CAE partially blocked nuclear translocation of NF-κB but activated the p53-associated apoptotic pathway.

Conclusion: These results indicate that the CAE has anti-melanoma potential, and the underlying mechanisms involve inhibition of the activities of NF-κB and its target proteins as well as promoting the activities of pro-apoptotic proteins.

1. Introduction

Malignant melanoma is one of the most aggressive human cancers. The worldwide incidence of melanoma is increasing more rapidly than that of any other cancer, as it has doubled in the past 10–20 years (Lens and Dawes 2004). Exposure to UV light, an irregular lifestyle and genetic factors are the major risk factors for the development of skin cancer (Coups et al., 2008). Among all skin cancer deaths, 75% are victims of melanoma, and more than 8000 individuals die annually in the United States (Lin et al., 2011; American Cancer Society, 2013).

Melanoma starts growing vertically from the basement membrane; therefore, the existence of a tumor is often detected late and only after it has become sufficiently large horizontally and fully vascularized (Pützer et al., 2010). After vascularization, the tumor grows rapidly, leading to metastasis; thus, in many cases surgical resection is impossible (Markovic et al., 2007). Therapeutic options are limited in patients with this highly metastatic disease, and the median survival period is only 6–9 months (Balch et al., 2003). A malfunctioning apoptotic mechanism in melanoma cells is the main reason for the poor response to conventional

Abbreviations: Bax, Bcl-2-associated X protein; Bcl-xL, B-cell lymphoma-extra large; Bcl-2, B-cell lymphoma 2; CAE, ethanol extract of *Cynanchi atrati* Radix; CCK-8, Cell Counting Kit-8; DAPI, 4,6-Diamidino-2-phenylindole; FACS, Fluorescence Activated Cell Sorting; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IKK-2 inhibitor IV, inhibitor kappa B kinase-2 inhibitor IV; IκBα, inhibitor kappa B alpha; Mdm-2, mouse double minute 2 homolog; NF-κB, nuclear factor kappa B; MEM-Eagle's salt, minimum essential medium-Eagle's salt; Noxa, phorbol-12-myristate-13-acetate-induced protein 1; PUMA, p53 up-regulated modulator of apoptosis; p53, protein 53; qPCR, real-time quantitative polymerase chain reaction

* Corresponding author. Tel.: +82 42 257 6397; fax: +82 42 257 6398.
E-mail address: ckson@dju.ac.kr (C.-G. Son).

http://dx.doi.org/10.1016/j.jep.2014.02.037
0378-8741

anti-cancer therapies (Helmbach et al., 2001; Grossman and Altieri, 2001). Excessive activity of nuclear factor kappa B (NF-κB) is related to resistance to chemotherapy and radiation during melanoma treatment (Amiri and Richmond, 2005).

Some medicinal plants or their active compounds have been investigated as novel candidate drugs to control melanoma development or growth (Lesiak et al., 2010; Kawano et al., 2007). *Cynanchi atrati* Radix (family Asclepiadaceae) has been traditionally prescribed for patients with tumor disorders or inflammatory fever, as described in the ancient medical text *Dong-ui-bo-gam* (UNESCO, 2009) for traditional Chinese and Korean medicine. One group demonstrated that *Cynanchi atrati* Radix inhibits the activities of several human cancer cell types (Day et al., 2001). Another group found that *Cynanchi atrati* Radix regulates apoptotic cell death by suppressing NF-κB signaling (Jeon et al., 2011). Based on these findings,*Cynanchi atrati* Radix was hypothesized to be an herbal drug that inhibits growth of melanoma.

In this study, we investigated the anti-melanoma effects of *Cynanchi atrati* Radix and its underlying molecular mechanisms using B16F10 cells.

2. Materials and methods

2.1. Preparation of Cynanchi atrati Radix ethanol extract (CAE) and compositional analysis

Dried *Cynanchi atrati* Radix was obtained from the Daejeon Oriental Medicine Hospital (Daejeon, Republic of K orea), and its identity was confirmed by a professional herbal pharmacist. The CAE was generated as follows. Briefly, 100 g of fully dried *Cynanchi atrati* Radix were pulverized with a grinder and mixed with 1 L of 70% ethanol on a moving shaker (150 rpm) for 24 h at room temperature. The supernatant was filtered through filter paper (Advantec, Dublin, CA, US) and centrifuged (4 °C, 500 rpm). The soluble fraction was condensed through a vacuum evaporator, which yielded 2.497 g of dried extract (final yield, 2.497%). The CAE was dissolved in distilled water before use and the remainder was stored at −70 °C for future use (VS No: LIRC-2013-02).

Fingerprinting to determine the reproducibility of the CAE and compositional analysis of putative compounds were conducted using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). A 20-mg aliquot of the CAE was dissolved in 1 mL of 90% methanol, and the solution was filtered (0.45 μm). The CAE sample solution was subjected to UHPLC–MS using an LTQ Orbitrap XL linear ion-trap MS system (Thermo Scientific Co., San Jose, CA, USA) equipped with an electrospray ionization source. Separation was performed on an Accela UHPLC system using an Acquity BEH C18 column (1.7 μm, 100 × 2.1 mm; Waters, Milford, MA, USA). The column was eluted at a flow rate of 0.4 mL/min using water (in 0.1% formic acid) and acetonitrile (in 0.1% formic acid), which were used as mobile phases A and B, respectively, with the following gradients: 0–1 min, 5% B (isocratic); 1–20 min, 5–70% B (linear gradient); 20–24 min, 70–100% B (linear gradient); 24–27 min, 100% B (isocratic). The CAE was detected using a photodiode array at 200–600 nm. The full-scan mass spectra were acquired at 150–1500 *m/z* in positive and negative modes. An Orbitrap analyzer was used for high-resolution mass data acquisition with a mass resolving power of 30,000 FWHM at 400 *m/z*. Tandem mass (MS/MS) spectra were acquired in data-dependent mode by collision-induced dissociation.

2.2. Chemicals and reagents

4,6-Diamidino-2-phenylindole (DAPI), formaldehyde, HEPES, potassium chloride, and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial antibodies were purchased from the following suppliers: anti-cytochrome *c* (Clontech, Palo Alto, CA, USA); anti-NF-κB p65 and anti-inhibitor kappa B alpha (IκBα) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-β-actin, anti-protein 53 (p53), anti-B-cell lymphoma 2 (Bcl-2), anti-Bcl-2-associated X protein (Bax), and anti-B-cell lymphoma-extra large (Bcl-xL) (Thermo Fisher Scientific Inc., Waltham, MA, USA); and anti-p53 up-regulated modulator of apoptosis (PUMA) (Epitomics AB, Burlingame, CA, USA). The inhibitor of inhibitor kappa B kinase (IKK), termed IKK-2 inhibitor IV, was purchased from EMD Millipore (Billerica, MA, USA).

2.3. Cell line and maintenance

The B16F10 cell line (murine melanoma cells) was purchased from the Korean Cell Line Bank (no. 80008, Seoul, Republic of Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin). The cells were maintained under humidified conditions at 37 °C in 5% CO_2. Some cells were sub-cultured and used in an *in vivo* tumor inoculation experiment, while the remainder was stocked to be used for further *in vitro* assay.

2.4. Animals and experiment design

Sixty specific-pathogen-free C57BL/6N male mice (age, 12 weeks; weight, 24–26 g) were purchased from Koatech (Gyeonggido, Republic of Korea). The mice were housed in a pathogen-free environment at 23 ± 2 °C under a 12-h light/dark day cycle. The animals were offered food and water *ad libitum* and were acclimatized for 1 week.

B16F10 cells (1×10^7 in saline) were injected subcutaneously into the right-lower flanks of each mouse using a 26-G needle. On day 15 after inoculation, mice with a palpable tumor mass were divided into three groups (n=20/group) by equalizing tumor size. Each mouse was orally administered with either distilled water (control group) or 100 or 200 mg/kg CAE (CAE 100 or CAE 200 groups respectively, CAE was dissolved in distilled water) for 10 days (daily). Body weights and tumor volumes of the animals were monitored every 2 days beginning on the first day of CAE administration. Additionally, the survival of mice in each group was monitored daily. On the last day of the experiment, all mice were sacrificed by dislocating the cervical vertebra. The tumors were isolated from the surrounding muscles and dermis. Tumor weights were measured with an electronic scale, and their volumes were measured using Vernier calipers (Maxwell Electronics, Vadodara, India). Tumor volumes were calculated based on the following formula: $(A \times B^2)/2$, where A is the larger and B is the smaller of the two dimensions. Isolated tumors were fixed in formalin for further study.

2.5. TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay for tumor tissues and B16F10 cells

The TUNEL assay was performed to detect apoptotic cells in tumor tissues and B16F10 cells using a TUNEL Apoptosis Detection Kit (Millipore). Briefly, tumor tissues were fixed in 10% formalin and embedded with paraffin. After deparaffinization and washing, the tissue specimens were incubated with Proteinase K at room temperature for 15 min. Then, 3% H_2O_2 was applied to quench any remnant peroxidase. After several washes, the specimens were incubated with TdT enzyme at 37 °C for 90 min followed by anti-digoxigenin-peroxidase treatment for 60 min at room temperature. 3-Amino-9-ethylcarbazole was used as the final chromogen during color development, along with hematoxylin counter staining.