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Apoptosis induced by caffeic acid phenethyl ester in human oral cancer cell lines: Involvement of Puma and Bax activation

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ABSTRACT

Objective: Caffeic acid phenethyl ester (CAPE), a natural honeybee product exhibits a spectrum of biological activities including antimicrobial, anti-inflammatory, antioxidant and antitumor actions. The purpose of this research was to investigate the anticancer potential of CAPE and its molecular mechanism in human oral cancer cell lines (YD15, HSC-4 and HN22 cells).

Design: To determine the apoptotic activity of CAPE and identify its molecular targets, trypan blue exclusion assay, soft agar assay, Western blot analysis, DAPI staining, and live/dead assay were performed.

Results: CAPE significantly suppressed transformation of neoplastic cells induced by epidermal growth factor (EGF) and 12-O-tetradecanoylphorbol 13-acetate (TPA) without inhibiting growth. CAPE treatment inhibited cell growth, increased the cleavages of caspase-3 and poly (ADP-ribose) polymerase (PARP), and augmented the number of fragmented nuclei in human oral cancer cell lines. CAPE activated Bax protein causing it to undergo a conformational change, translocate to the mitochondrial outer membrane, and oligomere. CAPE also significantly increased Puma expression and interestingly Puma and Bax were co-localized.

Conclusion: Overall, these results suggest that CAPE is a potent apoptosis-inducing agent in human oral cancer cell lines. Its action is accompanied by up-regulation of Bax and Puma proteins.

1. Introduction

Oral cancers are malignant lesions occurring in the oral cavity that include squamous cell carcinomas, salivary gland neoplasms, and odontogenic neoplasms (Krishna Rao, Mejia, Roberts-Thomson, & Logan, 2013) and are one of the most common causes of cancer-related mortality worldwide. Oral cancer is particularly dangerous because in its early stages it may not be noticed by the patient, as it can frequently progress without producing pain or recognizable symptoms and because it has a high risk of producing secondary tumors (Reis et al., 2011). Treatment often includes a combination of surgery, radiotherapy, and chemotherapy (Huang & O'Sullivan, 2013). Chemotherapy is strongly suggested to treat advanced-stage cancers. Recently, some vegetables, fruits, and grains that may offer substantial protection resources against various cancers as a practical therapeutic strategy have been investigated, especially for people at high risk for cancer (Chen, Hsieh, Chiou, & Chu, 2005; Vieira, de Souza, do Nascimento, & Leite, 2007). Therefore, it is important to make a scientific basis of these agents to achieve a successful chemotherapeutic outcome.

The honeybee products through mixing the secretions of their hypopharyngeal glands with resins collected from leaves and tree barks, which is used to make hives(S, 2014), propolis has been used in folk medicine to treat abscesses, canker sores, and for wounds (Tolba et al., 2014). Caffeic acid phenethyl ester (CAPE), one of the most extensively investigated active components of propolis, is a natural phenolic chemical compound (Russo, Longo, & Vanella, 2002). Its chemical name is 2-phenylethyl (2E)-3-(3,4-dihydroxyphenyl)acrylate and is also termed as phenylethyl caffeate or phenethyl caffeate (Murtaza et al., 2014). CAPE is a polyphenol with hydroxyl groups within the catechol ring

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which plays an important role in its biological activities (Wang, Stavchansky, Bowman, & Kerwin, 2006). Extensive research has been shown that CAPE has a multitude of beneficial biological properties including anti-inflammatory (Toyoda et al., 2009), antioxidant (Gocer & Gulcin, 2011), and anticancer activities (Juman et al., 2012). Particularly, it can induce apoptosis in a variety of cancer cell lines (Jin et al., 2008; Nomura, Kaji, Ma, Miyamoto, & Dong, 2001; Wang et al., 2005; Watabe, Hishikawa, Takayanagi, Shimizu, & Nakaki, 2004). Several studies have also shown anticancer activity effects of CAPE in oral cancer (Kuo et al., 2013; Lee et al., 2005). However, the exact molecular mechanisms underlying the anticancer activity of CAPE in the oral cancer cell lines have not been fully investigated yet. Thus, we evaluated the effects of CAPE on apoptosis and identified the underlying molecular signals related to CAPE-mediated apoptosis in various human oral cancer cell lines.

2. Materials and methods

2.1. Chemicals and antibodies

12-O-tetradecanoylphorbol-13-acetate (TPA) and 4′-6-Diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against cleaved PARP, cleaved caspase-3, Bax, Puma, Bad, Bim, Bak, Bcl-xL, and Mcl-1 were supplied by Cell Signaling Technology, Inc. (Beverly, MA, USA). Bax (6A7) antibodies were purchased from BD Pharmingen (San Jose, CA, USA). DyLight 488 AffiniPure Donkey Anti-Mouse IgG (H + L) was purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). Alexa Fluor® 488 Goat Anti-Rabbit IgG (H + L), Alexa Fluor® 594 Goat Anti-Rabbit IgG (H + L), and Cox4 antibody were obtained from Abcam (Cambridge, UK). Caffeic acid phenethyl ester (CAPE, Fig. 1A) and antibodies against actin and α-tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Cell lines and cell culture conditions

YD-15 (human mucoepidermoid carcinoma), HSC-4, and HN22 (human oral squamous cell carcinoma) cell lines were kindly provided by Yonsei University (Seoul, Korea), Hokkaido University (Hokkaido, Japan), and Dankook University (Cheonan, Korea), respectively. Murine skin epidermal cell line, JB6 was obtained from the American Tissue Culture Collection (Manassas, VA, USA). YD-15 cells were maintained in RPMI1640 medium and HSC-4 and HN22 cells were grown in DMEM; both types of media were supplemented with 10% fetal bovine serum (FBS). JB6 cells were cultured in MEM supplemented with 5% FBS. All cell lines were cultured at 37 °C in a 5% CO_2 incubator.

2.3. Trypan blue exclusion assay

To determine the number of viable cells, trypan blue dye (Gibco, Paisley, UK) was used. YD15, HSC-4, HN22 and JB6 cells were treated with DMSO or the designated concentrations of CAPE for 24 h. Cells were mixed with 0.4% trypan blue and counted with the hemacytometer and binocular microscope.

2.4. Anchorage-independent cell growth assay (Soft Agar Assay)

The inhibitory effect of CAPE on neoplastic transformation of JB6 cells was evaluated. Cells on 6-well plates were treated with TPA (20 ng/ml) or EGF (20 ng/ml) with or without various concentrations of CAPE (0.25 and 0.5 μM) in 1 ml of 0.33% basal medium Eagle's (BME) agar over 1 ml of 0.5% BME agar containing 10% FBS. The cell colonies were cultured at 37 °C in 5% CO_2 for 28 days, stained, and counted by image analysis software.

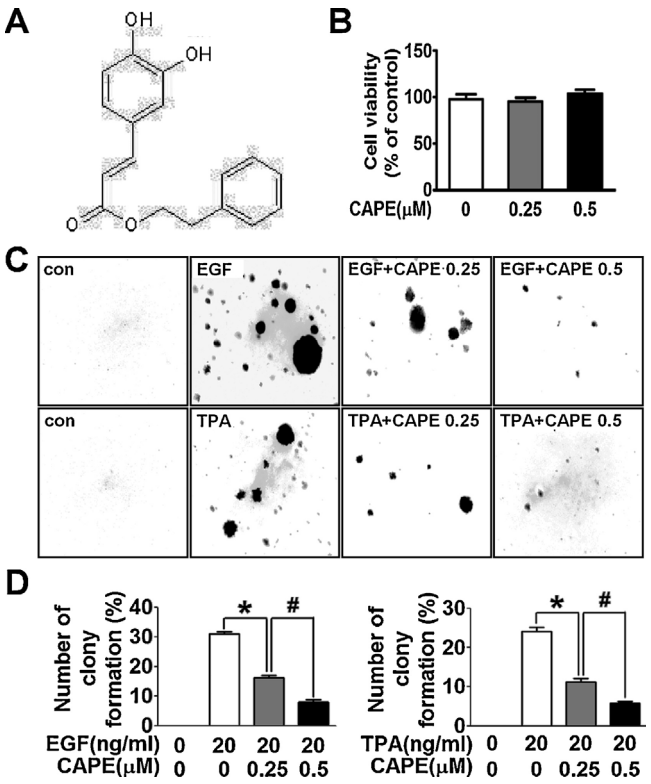

Fig. 1. Effects of CAPE on neoplastic transformation of EGF- or TPA-stimulated JB6 mouse skin epidermal cells (A) Chemical structure of CAPE. (B) JB6 cells were treated with DMSO or CAPE for 24 h. Cell viability was determined with a trypan blue exclusion assay. (C) For the soft agar assay, JB6 cells stimulated with TPA (20 ng/ml) or EGF (20 ng/ml) in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS were treated with CAPE and incubated at 37 °C in a 5% CO_2 incubator for 28 days, then colonies were counted. (D) Graphs express as means ± S.D. of triplicate experiments. *, $P < 0.05$, significance compared with the DMSO group.

2.5. Western blot analysis

Cells were scraped from culture dishes in lysis buffer and lysates were centrifuged for 10 min at 4 °C and 13,000 rpm. Protein concentration was determined with a DC Protein Assay kit (BIO-RAD Laboratories). For Western blotting, equal protein concentrations were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST buffer at RT for 1.5 h and probed with designated primary antibodies. After extensive washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 2 h. The antibody-bound proteins were detected with an ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.). Immunoreactive bands from three independent trials were captured and observed with an Image Quant LAS500 Imaging system (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.6. DAPI detection of apoptosis

Apoptosis was determined morphologically (by chromatin condensation and nuclear fragmentation) with a fluorescent nuclear dye, DAPI. Cells were treated with DMSO or CAPE (20 μM) for 24 h, then trypsinized, and fixed in 100% ethanol at −20 °C overnight. The cells were resuspended in PBS, deposited on poly-L-lysine coated slides, and stained with a DAPI solution (2 μg/ml). Cell morphology was observed under a fluorescence microscope (Leica microsystems, Wetzlar, Germany).