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Original Article

Phytochemical-induced reactive oxygen species and endoplasmic reticulum stress-mediated apoptosis and differentiation in malignant melanoma cells

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ABSTRACT

Background: Phytochemicals are derived from plants, vegetables and daily products and exert chemopreventive effects. Malignant melanoma is highly metastatic, and melanoma patients can develop chemotherapeutic resistance against conventional melanoma therapies.

Methods: In the present study, we investigated the anti-cancer effect of the phytochemicals kaempferol (Kaem), genistein (Gen), and 3′3-diindolylmethane (DIM) on melanoma cell viability. We also evaluated the altered expression of cell cycle-related genes. We verified the production of intracellular reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress at both the protein and cellular level using a western blot, TUNEL assay, and Dihydrodichlorofluorescein diacetate (DCF-DA) assay.

Results: Treatment of A375SM melanoma cells with phytochemicals resulted in inhibition of cell growth. Treatment with phytochemicals increased the gene expression of p21 and decreased the gene expression of cyclin E and/or cyclin B. The three phytochemicals activated the ROS-p38-p53 apoptotic pathway by increasing the level of phosphorylated p38 MAPK and p53, and they activated the ER stress-mediated apoptotic pathway by increasing the level of phosphorylated eIF2α and C/EBP homologous protein (CHOP). Both the ROS-p38-p53 and ER stress-mediated pathway induced the mitochondrial apoptotic pathway by attenuating Bcl-2 expression and upregulating BAX. Detection of morphological changes demonstrated that Kaem and Gen can induce differentiation in A375SM cell line.

Conclusion: These results indicate that phytochemicals are potentially useful in treatments for melanoma due to their ability to inhibit melanoma cell growth and division via the ROS and ER stress pathway.

Introduction

The prevalence of malignant melanoma, an exceptionally lethal form of skin cancer, has risen over the last few decades. Though it has well-defined diagnostic criteria and can be easily removed with a proper surgical procedure, cases that involve prior metastasis to distant sites have a low survival rate (Heo et al., 2016). Malignant melanoma occurs due to aggregation of multiple risk factors (extrinsic and endogenous). Mutations in genes related to critical growth factor signaling pathways in malignant melanoma such as ERK/MAP-kinase and PI3K/Akt are already widely known (Vogelstein and Kinzler, 2016; Wellbrock and Arozarena, 2016). The treatment for early stages of melanoma is surgical removal, and chemotherapy and radiation therapy are used for cases with prior metastasis; however, the latter therapies exhibit a low response rate. There have been many clinical trials focused on molecular-targeted and immunotherapies for malignant melanoma patients, but some patients developed resistance to these treatments.

Phytochemicals exhibit antioxidant properties and chemoprevention against cancers (Hwang and Choi, 2015; Liu, 2004). Humans consume phytochemicals in their daily lives through foods such as vegetables, legumes, fruits, and teas (Moon et al., 2006). Phytochemicals include genistein (Gen), kaempferol (Kaem) and 3′3-diindolylmethane (DIM). Genistein (Gen) and kaempferol (Kaem) are examples of isoflavones, which are present in soybeans, mung beans, and red clover. Kaem is a flavonoid with one hydroxyl group and is found in high concentrations in grapes, berries, and broccoli (Kim and Choi, 2013). It is also known to have anti-bacterial, anti-cancer, and anti-inflammatory properties (Lee et al., 2017). Gen is a typical isoflavone in the human diet and has shown an anti-tumor, anti-carcinogenic, and chemopreventive effect on tumors (Hwang et al., 2013). 3′3-diindolylmethane (DIM) is a natural compound produced as a metabolite of digestion of

Abbreviations: Kaem, kaempferol; Gen, genistein; DIM, 3′3-diindolylmethane; ROS, reactive oxygen species; ER, endoplasmic reticulum; EMT, epithelial-mesenchymal transition; CHOP, transcription factor C/EBP homologous protein; DCF-DA, dihydrodichlorofluorescein diacetate

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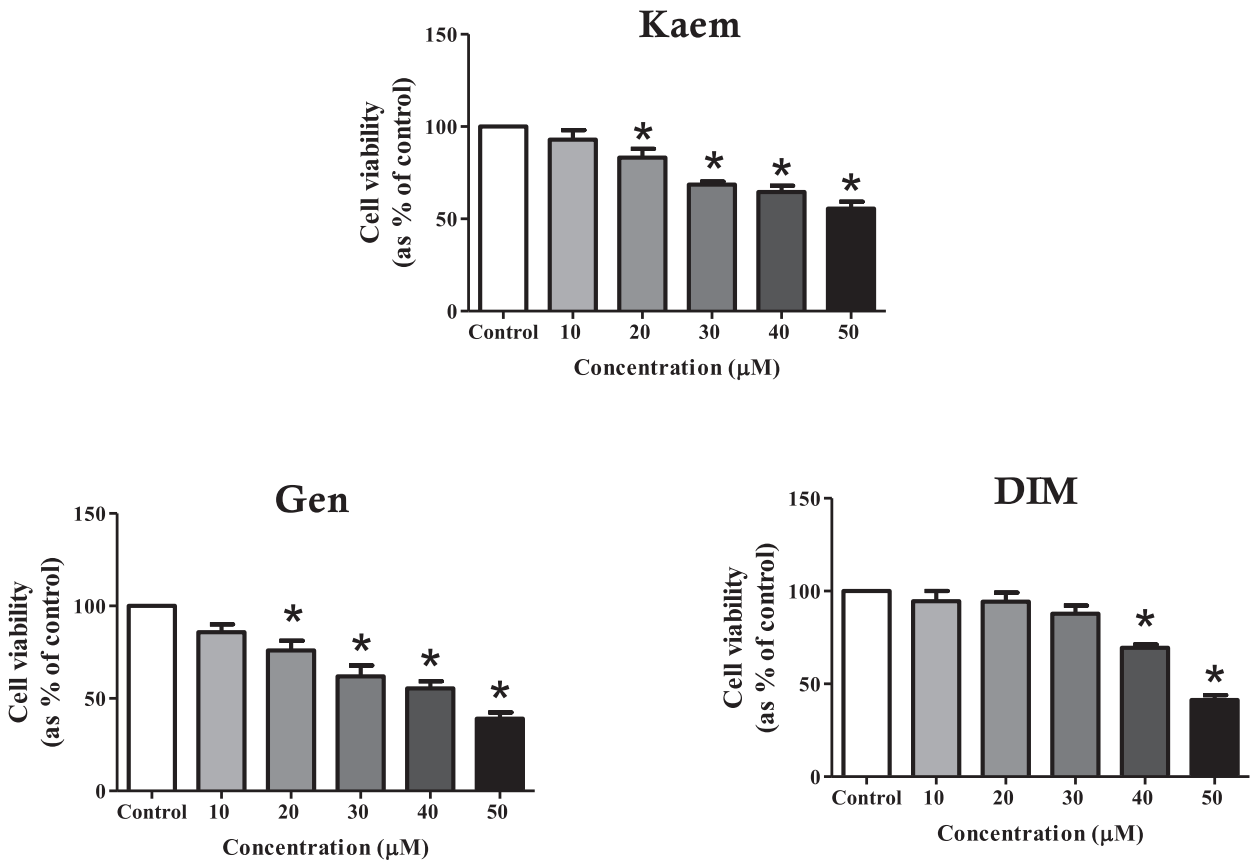

Fig. 1. ***Effects of three phytochemicals on cell viability of melanoma cell line.*** A375SM cells (3×10^3 cells/well) were seeded in 96 well plates. After 24 h, A375SM cells were treated with a medium containing 0.1% DMSO (control) and three phytochemicals (Kaem, Gen, and DIM) from 10 to 50 μM. On the last day of treatment, EZ-cytox was added to every well to quantify the cell viability. 0.1% DMSO was used as a control. Data were represented as mean ± SD. *, $P < .05$ vs. control (0.1% DMSO).

indole-3-carbinol, which is present in cabbage, Brussels sprouts, and kale (Boakye et al., 2016). DIM has displayed an anticancer property through induction of programmed cell death by regulating the apoptosis-related factors Bcl-2 and BAX. Moreover, it activates a mediator of the cell cycle and impedes metastasis progress in human breast cancer cells (Lee et al., 2016).

Exorbitant formation of reactive oxygen species (ROS) can cause oxidative stress, which results in cell damage that can lead to apoptosis (Poljsak et al., 2013). ROS is comprised of oxygen containing molecules such as superoxide, singlet oxygen, hydrogen peroxide, hydrogen peroxide, and hydroxyl radical (Riley, 1994). Oxidative stress is caused by an imbalance of pro-oxidants and antioxidants and can initiate change (Sies, 1991). For example, a few recent studies illustrated that consumption of antioxidants can raise mortality (Poljsak et al., 2013) and that large quantities of antioxidants can act as a prooxidant by enhancing oxidative stress (Podmore et al., 1998). Furthermore, high levels of antioxidants may break the balance between ROS formation and neutralization (Poljsak et al., 2013). Also, there were several studies illustrating that phytochemicals such as arctigenin and isoobtusilactone A induced ROS production by triggering an imbalance in the redox status of breast cancer cells, which induced the p38 MAPK and mitochondrial apoptotic pathways (Hsieh et al., 2014; Kuo et al., 2007).

The endoplasmic reticulum (ER) maintains the homeostasis of cellular proteins through mediation of protein synthesis and transport. ER stress occurs when unfolded or misfolded proteins accumulate in the ER and triggers many stress signaling pathways including apoptosis (Tabas and Ron, 2011). In addition, ER stress may trigger intracellular ROS generation, which intensifies ER stress-mediated apoptosis (Cao and Kaufman, 2014). One ER stress-induced apoptosis pathway involves activation of the transcription factor C/EBP homologous protein (CHOP) and attenuation of anti-apoptotic factors such as Bcl-2 and Bcl-xL to promote apoptosis (Marciniak et al., 2004). A few studies demonstrated that resveratrol, which is a type of natural phytoalexin produced by grapes, mulberries, and peanuts, etc., is able to induce apoptosis in multiple cancer cells by activating ER-stress (Gu et al., 2016; Rojas et al., 2014; Wang et al., 2011).

In the current study, we utilized three types of phytochemicals: Kaem, Gen, and DIM to evaluate and contradistinguish their effect on cell cycle and growth, ROS production, and ER stress-mediated apoptosis on a melanoma cell line.

Materials and methods

Reagents and chemicals

Kaem (≥97.0%) and DIM (≥98.0%) were bought from Sigma-Aldrich Corp. (St. Louis, MO, USA) and Gen (≥99.0%) was purchased from LC laboratories (Woburn, MA, USA). All chemicals were dissolved in dimethyl sulfoxide (DMSO; Junsei Chemical Co., Tokyo, Japan).

Cell culture and media

A malignant melanoma cell line, A375SM, was purchased from Korean Cell Line Bank (KCLB, Seoul, South Korea) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; RMBIO), 10 U/ml penicillin and 100 μg/ml streptomycin (Cellgro Mediatech, Manassas, USA), and 10 mM HEPES (Invitrogen Life Technologies, Carlsbad, USA) at 37 °C in 5% CO_2 and 95% air in a humidified cell incubator. 0.05% trypsin/0.02% EDTA (PAA Laboratories, Dartmouth, MA, USA) was utilized for trypsinizing the cells.