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Naringin induces death receptor and mitochondria-mediated apoptosis in human cervical cancer (SiHa) cells

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ABSTRACT

Cervical cancer is the second most common female cancer worldwide, and it remains a challenge to manage preinvasive and invasive lesions. Fruit-based cancer prevention entities, such as flavonoid and their derivatives, have demonstrated a marked ability to inhibit preclinical models of epithelial cancer cell growth and tumor formation. Here, we extend the role of naringin-mediated chemoprevention to that of cervical carcinogenesis. The present study sought to investigate the therapeutic potential effect of naringin on apoptosis in human cervical SiHa cancer cells. Viability of SiHa cells was evaluated by the MTT assay, apoptosis and mitochondrial transmembrane potential by flow cytometry, and pro-apoptotic related genes by Real-time quantitative PCR. Naringin showed a 50% inhibition of SiHa human cervical cancer cells at a concentration of 750 μM. SiHa cells exhibited apoptotic cell death, intranucleosomal DNA fragmentation, morphological changes and decline in the mitochondrial transmembrane potential. In addition, administration of naringin increased the expression of caspases, p53 and Bax, Fas death receptor and its adaptor protein FADD. These results suggest that the induction of apoptosis by naringin is through both death-receptor and mitochondrial pathways. Taken together, our results suggest that naringin might be an effective agent to treat human cervical cancer.

1. Introduction

Cervical carcinoma is the second most common female cancer worldwide. It is estimated that 12,200 new cases and about 4210 deaths will be attributed to cervical cancer in the United States in 2010 (American Cancer Society, 2010). Cervical cancer, the seventh most frequent women cancer in Saudi Arabia and the eighth most common cancer among women aged between 15 and 44 years (WHO, 2007). It is generally accepted that radical surgery or radiotherapy can be curative for the majority of patients with early-stage cervical cancer, while chemotherapy or neoadjuvant chemotherapy is always the first choice for those patients with advanced cervical cancer, where the prognosis remains very poor (Thomas, 1999; Ren et al., 2008). Therefore, many researchers have been trying to find more effective chemotherapeutics to treat cervical cancer cell. Apoptosis is an active form of cell suicide controlled by a network of genes and it is an essential process, as well as a key role, in the pathogenesis of diseases including cancer (Li et al., 2009). During the past decade, numerous reports have proven that many cancer chemotherapeutic agents kill the cancer cell by inducing apoptosis (Mandal et al., 2010; Kim et al., 2010. Thus, clarification of the induction mechanisms of cell apoptosis is useful to approach in cancer therapies.

Epidemiological evidence also indicates a high intake of fruits and vegetables leads to a reduction in cancer incidence, and it is the phytochemicals within fruits and vegetables that have been proposed as responsible for their protective effects (Kroon and Williamson, 2005). Consequently, in the past few decades chemoprevention through dietary phytochemicals has become an increasingly active area of research and entails using non-toxic substances to interfere with carcinogenesis (Surh, 2003; Johnson et al., 2010). Flavonoids, a family of natural polyphenolic compounds, are commonly found in fruits and vegetables, regularly consumed by humans. Flavonoids are composed of several classes including flavonols, flavonones, flavones, flavanols, iso-flavonoids and antho-cyanidins. They have been demonstrated to possess anti-cancer and chemopreventive property in numerous epidemiological studies (Park and Surh, 2004; Ramos, 2007; Arts, 2008; Miller et al., 2008). At the cellular and molecular level, flavonoids have a broad range of effects and interactions that contribute to their chemopreventive and anti-carcinogenic activities. The modulation of cellular processes such as cell cycle and apoptosis contributes to their anti-proliferative effects (Kuntz et al., 1999;

Abbreviations: DMSO, dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; ψM, mitochondrial transmembrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide.

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Agarwal, 2000). In addition, flavonoids interact with receptors, enzymes, and kinases in their molecular levels (Gamet-Payrastre et al., 1999; Collins-Burow et al., 2000; Manthey et al., 2001; Jacobson et al., 2002).

Naringin is a kind of bioflavonoid derived from grapefruit and related citrus species (Jagetia and Reddy, 2002). Naringin or its metabolite naringenin has been reported to possess diverse biological and pharmacological properties including anticarcinogenic (So et al., 1996), lipid-lowering (Jeon et al., 2004), superoxide scavenging (Rajadurai et al., 2009), anti-apoptotic (Kim et al., 2009), anti-atherogenic (Choe et al., 2001), metal chelating (Jagetia et al., 2003) and antioxidant activities (Jagetia and Reddy, 2005). Recently, growing evidence has indicated that naringin or naringenin displays anti-inflammatory effects both in *in vitro* and *in vivo* systems (Kanno et al., 2006; Amaro et al., 2009; Lee et al., 2009; Ribeiro et al., 2009; Shi et al., 2009). Fig. 1 depicted the structure of naringin. In recent years, experiments have been performed on supplementation with natural flavanones, especially compounds found in vegetables, fruits and medicinal plants, have received increasing attention for their potential role in prevention of cancer (Szliszka and Krol, 2011). Therefore, this study will be designed to investigate the effects of flavanones on the cell viability, oxidative stress, and expression of caspases, and p53 in SiHa cancer cells. In the present study, naringin was shown to kill human cervical cancer cells preferentially. Apart from inhibiting cell proliferation, naringin was also found to induce apoptosis in human cancer cells through both death receptor and mitochondrial pathways. The results are significant as they provide new insights in understanding the molecular mechanism of naringin that might be a potent chemotherapeutic agent for the treatment of human cervical cancer.

2. Materials and methods

2.1. Materials

Naringin, propidium iodide, (PI), acridine orange and ethidium bromide were purchased from Sigma–Aldrich chemical co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide (JC-1) and Dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The DeadEnd TUNEL assay kit was purchased from Promega (Madison, USA). Annexin V-Cy3 apoptosis detection kit was procured from Abcam (Cambridge, MA, USA). QuantiTect Primer assay, FastLane Cell cDNA Kit, and QuantiFast SYBR Green PCR Kit were obtained from QIAGEN (Germany). All the other chemicals used were of research grade.

2.2. Maintenance of SiHa cells

The SiHa cervical cancer cell line was provided by Prof. M.A. Akbarsha at the Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animals in Life Science Education, Bharthidasan University, India. The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured as an adherent monolayer at approximately 70–80% confluence and at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested after brief trypsinization. Cells in the exponential growth phase were used in all experiments.

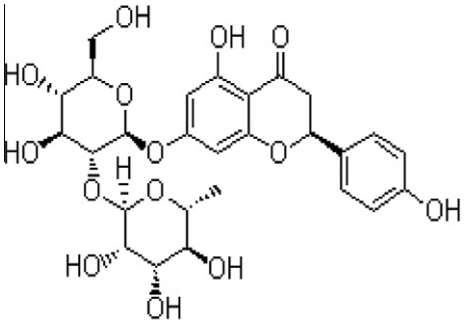

Fig. 1. Molecular structure of naringin. Naringin [7-(2-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyloxy)-2,3-dihydro-4′,5,7-trihydroxyflavone] CAS: 10236–47-2. Molecular formula: $C_{27}H_{32}O_{14}$; molecular weight: 580.50.

2.3. Preparation of naringin

A stock solution of 20 mg/ml was prepared in DMSO (Sigma, USA). The solution was stored in aliquots at −20 °C. Further dilutions were made in DMEM to required concentrations between 250 and 2000 μM for the treatment of SiHa cells.

2.4. Measurement of cell viability

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazon product (Mosmann, 1983). Briefly, SiHa cells were plated at a density of 2.5×10^3 cells/ml in 96-well plates. After overnight growth, cells were treated with a 250–2000 μM of naringin for 24/48 h. Subsequently, the cells were washed with 200 μL of PBS, and incubated with 100 μl of 500 μg/ml MTT in PBS at 37 °C for 3 h. The MTT-formazon product dissolved in 200 μl of DMSO was estimated by measuring the absorbance at 570 nm in a Bio-Rad multiwell plate reader and the IC_{50} was calculated. Quadruplicate samples were run for each concentration of naringin in three independent experiments. The cell viability of SiHa cell line was expressed as the percent viability of treated cells compared with the untreated control.

2.5. Analysis of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential ($\Delta\psi M$) were determined using JC-1, a fluorescent carbocyanine dye, which accumulates in the mitochondrial membrane as a monomer or dimer depending on the mitochondrial membrane potential (Smiley et al., 1991). Briefly, cells were plated at a seeding density of 5×10^4 cells/well in a 24-well plate. After 24 h of treatment with IC_{50} (750 μM) of naringin, cells were incubated with 2.5 μM JC-1 for 15 min at 37 °C in darkness. Subsequently, stained cells were washed with PBS, followed by FACS analysis (BD FACSCanto™ II, San Jose, CA, USA). The presence of JC-1 monomers or dimers was examined under a fluorescent microscope using filter pairs of 530 nm/590 nm (dimers) and 485 nm/538 nm (monomers).

2.6. Detection of apoptosis by fluorescence microscopy

The nuclear morphology was analyzed by treatment of SiHa cells with (750 μM) naringin for 24 h. Control cells were grown in the same manner in absence of naringin. Cells were trypsinized and fixed with methanol. Then, cell nuclei were stained by treatment with 1 mg/mL Propidium Iodide (Sigma) at 37 °C for 15 min in the dark. Characteristic apoptotic morphological changes were determined by acridine orange/ethidium bromide (AO/EB) staining as described by Leite et al. (1999). Stained cells were examined under a fluorescence inverted microscope (Carl Zeiss, Jena, Germany).

2.7. Analysis of the cell cycle distribution

SiHa cells were plated at 2×10^5 cells/ml in a six-well plate. After a 24 h incubation (37 °C, 5% CO2), the cells were treated with naringin prepared in serum-free media and incubated for an additional 24 h. The IC_{50} determined from the cytotoxicity assay for each treatment was used in the cell cycle assay. The cell cycle phase evaluation was performed as described by Grassi et al. (2007). Following trypsinization, cells were centrifuged at 1000 × g for 10 min, and the pellet was resuspended in 0.5 ml of PBS. Fixation was completed by adding 4.5 ml of 70% cold ethanol for at least 2 h. The fixed cells were centrifuged at 1000g for 10 min, and the pellet suspended in 5 ml of PBS. After 60 s, the cells were centrifuged as before, and the pellet resuspended in 1 ml of propidium iodide (PI) staining solution. Immediately after 15 min incubation at 37 °C, the cells were analyzed to determine the cell cycle stage using flow cytometry (BD FACSCantoTM II, San Jose, CA, USA) with an excitation wavelength of 488 nm and an emission at 670 nm. The data presented are representative of those obtained in at least three independent experiments conducted in triplicate.

2.8. Annexin V-Cy3 assay

The phosphatidylserine-binding protein Annexin V was conjugated to Cy3 using the commercially available Annexin V-Cy3 apoptosis detection kit with SYTOX (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. To examine whether cell death occurred via apoptosis or necrosis, SYTOX was used, which being a non permeable stain having affinity towards nucleic acids, selectively enters necrotic or late apoptotic cells. Therefore, double-staining of Ann-Cy3 and SYTOX helps discriminate between live cells (SYTOX and Annexin V negative), cells in early apoptosis (Annexin V positive, SYTOX negative), cells undergoing late apoptosis (both Annexin V and SYTOX positive) and necrotic cells (SYTOX positive, Annexin V negative). SiHa cells were plated in 25-cm^2 tissue culture flasks at 2×10^5 cells