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Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway

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ABSTRACT

Ovarian cancer is a significant malignancy for women in the Western world, and its death rate has remained unchanged over the past 50 years, leaving room for proper chemoprevention. Kaempferol is a natural flavonoid widely distributed in fruits and vegetables, and epidemiological studies have found a negative correlation between kaempferol consumption and ovarian cancer risk. To understand the mechanism behind this negative correlation, we investigated kaempferol's ability to induce apoptosis in A2780/CP70, A2780/wt, and OVCAR-3 ovarian cancer cell lines. Kaempferol inhibited cell proliferation but did not cause necrosis in all 3 cell lines. For the apoptosis, caspase 3/7 levels were induced in a concentration-dependent manner by kaempferol treatment, with A2780/wt cells being the most responsive. This induction can be diminished by pre-treatment with a caspase-9 inhibitor, indicating an intrinsic apoptosis pathway. Western blot analysis revealed that protein levels of Bcl-x_L were decreased in ovarian cancer cells, whilst p53, Bad, and Bax proteins were up-regulated by kaempferol treatment. Our data indicate that kaempferol induces apoptosis in ovarian cancer cells through regulating pro-apoptotic and anti-apoptotic protein expressions in the intrinsic apoptosis pathways, and is a good candidate for the chemoprevention of ovarian cancers in humans. Further studies in animal models and clinical trials are therefore warranted.

1. Introduction

It is estimated that 13,850 women in the United States will die from ovarian cancer in 2010, marking 5% of the total cancer deaths in females (Jemal, Siegel, Xu, & Ward, 2010). Prevention of ovarian cancer is challenging, because no specific carcinogen is known to cause this disease (Banks, 2000) and no specific biomarker is clinically available for screening and early diagnosis (Skates, Jacobs, & Knapp, 2000). Prevention of ovarian cancer is, however, possible, because migration studies found that this disease is more related to environmental factors than to genetic background (Banks, 2000). The question is which environmental factors or life styles can reduce the risk of ovarian cancers.

It is a common belief that a diet rich in fruits and vegetables will help reduce the risk of various chronic diseases, including cancers. More specifically, low intake of vegetables has been consistently associated with an increased risk of ovarian cancer (Banks, 2000). Kaempferol is a natural flavonoid that is widely distributed in fruits and vegetables, and prospective studies revealed that over decades, consumption of kaempferol dramatically and significantly reduced the risk of ovarian cancer in American female nurses (Gates et al., 2007). This finding suggests that kaempferol is a promising agent for the chemoprevention of ovarian cancers, because it is a dietary component, relatively non-toxic, inexpensive, and consumption of kaempferol can be easily adopted into the lifestyles of most women. The chemopreventive mechanism, however, is unclear or incomplete, and some basic mechanistic studies are needed before designating kaempferol as a real chemoprevention agent.

Our earlier studies have demonstrated that kaempferol inhibits expression of vascular epithelial growth factor (VEGF) and angiogenesis in ovarian cancer cells (Luo et al., 2009), and this effect will indirectly prevent ovarian cancer cells from demonstrating unlimited proliferation. However, kaempferol's direct effects on ovarian cancer cells are still unknown. Whilst short-term exposure to kaempferol does not cause any necrosis in ovarian cancer cells (Luo, Jiang, King, & Chen, 2008), long-term effects are unknown for both necrosis and apoptosis. Meanwhilst, kaempferol has been reported to induce apoptosis in some cells (Huang et al., 2010; Nguyen et al., 2003; Sharma et al., 2007), but inhibit apoptosis in other cells (Ruiz, Padilla, Redondo, Gordillo-Moscoso, & Tejerina, 2006). To promote kaempferol toward a chemoprevention agent, kaempferol's effects on ovarian cancer cells need to be better characterised and kaempferol's underlying mechanisms of action need to be examined. In this study, we investigated whether kaempferol

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treatment will cause necrosis and/or apoptosis in ovarian cancer cells, and the pathway involved for these effects.

2. Materials and methods

2.1. Cell culture and treatment

OVCAR-3 and A2780/CP70 ovarian cancer cell lines were provided by Dr. Jiang at the West Virginia University, and the A2780/wt ovarian cancer cell line was kindly provided by Dr. Kenneth Tew at the Medical University of South Carolina. IOSE 364, normal ovarian surface epithelial cells from healthy women, but immortalised with SV40 T/t, were courtesy of Dr. Auersperg at University of British Columbia, Canada. All cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C with 5% CO_2. A stock solution of kaempferol (Sigma) and cisplatin (Sigma) were prepared in dimethyl sulfoxide (DMSO) at 100 mM and stored at −20 °C. Different concentrations of kaempferol and cisplatin were prepared in a RPMI 1640 medium with FBS for cell treatments, and DMSO was included in the preparations to ensure equal concentrations of DMSO in each treatment.

2.2. Cell proliferation assay

As kaempferol has a yellow colour, a colour gradient appeared in serial dilutions of kaempferol in the RPMI 1640 medium, which interferes with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based colour absorbance assays (unpublished data). Repeated washing of cells with PBS, effectively removed colourful treatments, but caused an appreciable and an uneven loss of cells (unpublished data). For this reason, genomic DNA abundance was measured to estimate cell numbers after each treatment. Ovarian cancer cells were seeded in 96-well plates at 2000 cells/well and incubated overnight before treatment with 0–160 μM kaempferol for 24 h in triplicates. The medium was removed, and the plates were freeze–thawed to lyse cells. Each well was added with 200 μl 1× CyQUANT cell lysis buffer (Invitrogen) containing 5× SYBR Green I (Invitrogen) and incubated at room temperature (RT) for 5 min. The reaction (50 μl) was transferred to PCR strip tubes and the fluorescent signal was measured at 90 °C with a real-time Chromo4™ PCR instrument (Bio-Rad, Hercules, CA). To ensure that cell proliferation assays were performed within a linear range of cell numbers, a standard curve was generated by seeding different amount of OVCAR-3 cells (based on counting with a haemocytometer) in a 96-well plate, and measuring genomic DNA abundance after overnight incubation. Three independent experiments were performed and data was pooled for statistical analysis.

2.3. Cytotoxicity assay

Ovarian cancer cells were seeded in 96-well plate at 5000 cells/well, incubated overnight, and treated in triplicates with 100 μl kaempferol for 24 h. Culture medium (20 μl) was sampled to measure free lactate dehydrogenase (LDH) levels. Lysis solution (10×) was then added into cells and incubated at 37 °C for 1 h to release all LDH into culture medium, which is sampled again (20 μl) to measure total LDH levels. A CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) was deployed to measure the free and total LDH levels from each well, and non-cell wells were measured for background control. LDH levels contained in intact cells were derived by subtracting the free LDH levels from total LDH levels, and normalised to the total LDH of the control samples. Two to three independent experiments were carried out and the results pooled for statistical analysis.

2.4. Apoptosis assay

To time apoptosis in ovarian cancer cells, A2780/CP70 ovarian cancer cells were seeded in 96-well plates at 10,000 cells/well, incubated overnight, and treated in triplicates with 0- or 80-μM kaempferol for 0, 2, 4, and 8 h. No-cell wells were included for background correction. At each time point, a plate of the culture medium was removed and frozen in −80 °C until final analysis. Cells were thawed, lysed with Passive Lysis Buffer (Promega), and analysed for caspase 3/7 activities with a Caspase-Glo 3/7 Assay (Promega) and the total protein levels with a BCA assay (Pierce) as per the instructions. Caspase 3/7 activities were normalised by total protein levels, and the levels of kaempferol-treated cells were expressed as percentages of controls for statistics. To analyse kaempferol-induced apoptosis in ovarian cells, all 3 lots of ovarian cancer cells and 1 lot of immortalised normal cells were seeded in 96-well plates, incubated overnight, and treated with various concentrations of kaempferol for 2 h. A positive control, cisplatin, was also included in the cancer cell treatments for confirmation and comparison. Caspase 3/7 activities and total protein levels were analysed as described above.

2.5. Caspase-9 inhibition experiment

OVCAR-3 cells were seeded in 96-well plates at 10,000 cells/well and incubated overnight. Cells were treated with a caspase-9 inhibitor (Z-LEHD-FMK) (0–10 μM) or 10 μM negative control (Z-FA-FMK) (Biovision, Mountain View, CA) in triplicates for 24 h, and treated with 0 or 80 μM kaempferol for 2 h. Caspase 3/7 activities were measured with a Caspase-Glo 3/7 Assay (Promega) and normalised by cell numbers, which were measured with a CellTiter 96 Aqueous One Solution Cell proliferation Assay (Promega) following the manufacturer's instructions. Data from two independent experiments were pooled for statistical analysis.

2.6. Western blot

Ovarian cancer cells were seeded in 60-mm dishes, incubated overnight, and treated with 0–80 μM kaempferol for 24 h. The cells were harvested with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Pierce) as per the instructions, subject to SDS–PAGE, transferred to nitrocellulose membranes, blocked with 5% non-fat milk in TBST, and probed with different primary antibodies targeting human p53, Bad, Bax, or Bcl-x_L (Santa Cruz, CA). Membranes were incubated with Goat-anti-Mouse-Poly-HRP (Pierce) and visualised with X-ray film exposure. Membranes were further stripped with Restore PLUS Western Blot Stripping Buffer (Pierce) and re-probed with a GAPDH antibody (Santa Cruz) to normalise target protein abundances. Film images were quantitated with NIH "ImageJ" software and 2–3 independent experiments were pooled for statistical analysis.

2.7. Statistical analysis

All replicates within an experiment were averaged and the mean values from different experiments were pooled for statistical analysis. One-way ANOVA followed by a Dunnett's test or *t*-test was performed, as appropriate, to determine the differences between groups using SPSS software and a *p*-value of less than 0.05 is considered to be significant.