ARTICLE

Inducement of apoptosis by cucurbitacin E, a tetracyclic triterpenes, through death receptor 5 in human cervical cancer cell lines

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Cervical cancer is the most common malignancy in women, for which conization or hysterectomy are the main therapy. Curcubitacin E (Cu E) is a natural compound-based drug which from the Guadi (climbing stem of Cucumic melo L). Previously shown to be an anti-tumor as well as a potent chemopreventive agent against several types of tumors. The present study, investigated anti-proliferation and apoptosis induced by Cu E in cervical cancer cell lines (HeLa and Ca Ski). The results indicate that the cytotoxicity is associated with accumulation in apoptosis but not necrosis. Cu E produced apoptosis as well as the up-regulation of death receptor 5 (DR5). In addition, the DR5 gene activation in apoptosis, both effects increased proportionally with the dose of Cu E; however, mitosis delay was also dependant on the amount of Cu E treatment in the cancer cells. These results indicate that Cu E may delay cancer cell growth by apoptosis via upregulation of DR5 gene expression.

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INTRODUCTION

Cervical cancer is the sixth most common cancerous malignancy in females in Taiwan, and a leading cause of death among gynecological malignancies.1 The majority of women diagnosed with this cancer exhibit an advanced, widely disseminated malignancy and poor survival rate.2 Infections with human papillomavirus (HPV), predisposition, as well as various factors are believed to have important roles in the development of carcinogenesis.3 Overwhelming evidence has demonstrated that oncogenic types of HPV play an important role in the development of precursors of cervical cancer.4 However, only a small fraction of females infected with HPV develop the disease, indicating the contribution of other factors to the progression of lesions in invasive cervical cancer.5 Many studies indicate a positive correlation between the consumption of natural components of certain plants and the decreased incidence of some tumors including prostate, cervical, ovarian, lung and gastrointestinal tract.6–7 Cucurbitacins are a group of tetracyclic triterpenes with medicinal properties derived from the climbing stem (Gua di) of Cucumic melo L.8 Gua di has been used extensively in traditional folk medicines throughout Asia, providing selective biological activities.9 Interest in this herb has grown in recent years due to its putative beneficial pharmacological effects as an anti-inflammatory10 and anti-cancer agent.11 There have also been indications that cucurbitacins may help to prevent and treat oxidative damage as well as suppress specific inflammatory factors.12

Cucurbitacin E (Cu E) is an active compound, which was previously shown to be a strong anti-feedant with the ability to disrupt cell actin and adhesion.13 Recent our studies have reported that Cu E has an inhibitory effect on the proliferation of cancer cells.14 However, its remains unclear whether Cu E inhibits the growth of cervical cancer cells. Furthermore, the mechanism underlying the anti-cancer effects of Cu E has yet to be identified.

This present study was initiated to investigate whether Cu E contributes to the anti-proliferation and apoptosis induction of cervical cancer cell lines (HeLa and Ca Ski). It is expected that these experiments will provide scientific basis and technological support for further development of cervical cancer therapy.

RESULTS

Cu E can mediate the survival of cervical cancer cells, and thus inhibits their growth

To explore this anti-tumor activity, an in vitro study was conducted in which HeLa and Ca Ski cells were subjected to increasing dosages of Cu E (0, 1.25, 2.5 and 5 µM) for 1 to 3 days. The proliferation of Cu E-treated cancer cells were then measured using the MTT method (Figure 1a). The results indicate that the survival and proliferation of Hela and Ca Ski cells was decreased by Cu E treatment in a dose- and time-dependent manner. (HeLa: \( R = 0.9077 (24\ h) \), \( y = -16.585x + 107.48, R^2 = 0.8304 \) (48 h); \( y = -20.23x + 118.58, R^2 = 0.9672 \) (72 h); Ca Ski: \( y = -12.309x + 109.15, R^2 = 0.9432 \) (24 h); \( y = -17.794x + 108.13, R^2 = 0.8341 \) (48 h); \( y = -18.951x + 107.19, R^2 = 0.7949 \) (72 h)).

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Cu E-induced apoptosis of cervical cancer cell lines
To identify the role played by Cu E in the apoptosis/necrosis of cervical cancer cell lines, we employed Annexin V-FITC and propidium iodide staining to reveal the formation of apoptotic cells following 4 h of exposure to Cu E. The percentage of apoptotic cells was assessed by flow cytometric analysis (Figure 1b). A dot-plot of Annexin V-FITC fluorescence versus PI fluorescence indicates a significant increase in the percentage of apoptotic cells treated with Cu E, compared with untreated cells. Significant increase was observed in the percentage of cells undergoing apoptosis (Figure 1c).

Cu E-induced accumulation of Sub G1 phase in Cu E-treated cells
The cell cycle distribution of Cu E-treated cells was analyzed by flow cytometry. Cells were exposed to Cu E for 24 h before processing and analysis. As shown in Figure 2a, exposure to Cu E resulted in an increase in the number of G2/M phase and sub G1, cells, which may imply that the cervical cancer cells underwent cell cycle arrest and apoptosis. The results indicate that treatment with Cu E increased the cell populations in sub G1 (HeLa: \( y = 2.5136x + 3.1373, R^2 = 0.8259 \); Ca Ski: \( y = 0.7428x + 5.4593, R^2 = 0.1811 \)), while simultaneously reducing the number of cells in the G1 phases in HeLa cells (\( ^*p < 0.05 \) versus Cu E 0 \( \mu M \)) \((y = -3.6922x + 51.15, R^2 = 0.244)\) (Figure 2b).

Assessment of changes in mitochondrial membrane potential
The loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event coinciding with caspase activation. In non-apoptotic cells, JC-1 exists as a monomer in the cytosol (green) and accumulates as aggregates in the mitochondria, which appear red. In apoptotic and necrotic cells, JC-1 exists in monomeric form and stains the cytosol green. Figure 2c shows typical FL-1/FL-2 dot plots for JC-1 staining HeLa and Ca Ski cells with apoptosis. Cu E-free cancer cells are without apoptosis, which have red fluorescing J-aggregates. The green fluorescing monomers shown in the lower part indicate apoptotic cells. Figure 2d shows the percentages of apoptotic cells analyzed by flow cytometer in different Cu E-treated groups. Taken together, the observations imply that Cu E has significantly reduced the mitochondrial membrane potential of HeLa and Ca Ski cells. Moreover, we detected caspase 3 activation at Cu E concentrations of 1.25 to 5 \( \mu M \) in HeLa \((y = 3.3754x - 1.0423, R^2 = 0.8835)\) and Ca Ski cells \((y = 1.9717x + 3.0832, R^2 = 0.8243)\) (Figures 2e and f), indicating induction of apoptosis in treated cells. There are also significant change was observed in the pro-caspase 3, -8 and -9 in Cu E-treated Ca Ski cells (Figures 3a and b).

In summary, the results summarized in Figures 1–3 suggest that Cu E affects the survival of cervical cancer cell lines inducing mitochondrial depolarization and apoptosis.
Apoptosis induction in Cu E-treated cells via DR5 upregulation

Figures 4a–c illustrate DR5 gene expression in cervical cancer cells (Figure 4a) and immunoblotting results of cellular proteins from HeLa and Ca Ski cell lines treated with Cu E (Figure 4d). Gene expression and western blot analysis revealed an increase in DR5 following incubation with Cu E (Figures 4d and e).

These data suggest that DR5 level regulated the tumorigenicity of cervical cancer cells via Cu E. These findings indicate that common molecular pathways are involved in inducing apoptosis. Findings from qPCR analysis were further validated by a microarray analysis (data not shown), which indicated substantial TNF up-regulation (relative expression ratio 3.08) as well as...
**MATERIALS AND METHODS**

**Materials**

Cucurbitacin E, DMSO (dimethyl sulfoxide) and MTT (3-[4,5-dimethylthiazol-2-yl]–2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich (St Louis, MO, USA). Cell culture medium (MEM and RPMI 1640), phosphate-buffered saline (PBS), anti-biotics, sodium pyruvate, L-glutamine, trypsin and fetal bovine serum (FBS) were purchased from Gibco, BRL (Grand Island, NY). Polyvinylidene fluoride membrane (PVDF) (Millipore), and molecular weight marker were purchased from Bio Rad (USA). All other reagents and compounds were analytical grades.

**Cells**

The HeLa and Ca Sk cells were purchased from ATCC. The cells were maintained on culture dishes, in 90 % (v/v) MEM Eagle or RPMI1640 with 2 mM L-glutamine and Earle’s BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acid (NEAA) and 1 mM sodium pyruvate with 10% (v/v) FBS. The cells were cultured in an atmosphere containing 5% CO2 at 37 °C incubator.

**Cell proliferation assay**

The cancer cells were seeded into 96-wells culture plate at 5000 cells/well. The cells were treated with 0, 1.25, 2.5 and 5 μM Cu E, the Cu E will complex with culture medium. Then the cell will incubate in 37 degree C for 24, 48 and 72 h in the CO2 incubator. After incubate 24, 48 and 72 h. The cell treatments MTT dye (1 mg/ml) at least 4 h on each well. The reaction was stopped by the addition of DMSO, and optical density was measured at OD540 on a multi-well plate reader. Background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean for each experiment was calculated. Results were expressed as a percentage of control, which was considered as 100%. Each assay was carried out in triplicate and the results were expressed as the mean (±S.E.M.).

**Measurement of apoptosis/necrosis**

Cervical cancer cell lines were first seeded in 6-well plates (Orange Scientific, E.U.). Following treatment with Cu E for 4 hours, the cells were harvested. The cells were re-centrifuged (the supernatant discarded) and resuspended/incubated in 1X annexin-binding buffer. Five microliter of annexin V-FITC (BD Pharmingen, Franklin Lakes, NJ, USA) and 1 μl of 100 μg/ml PI working solution for 15 min. Following the incubation period, the stained cells were analyzed using flow cytometry (FACSCalibur, BD, San Jose, CA, USA). Data was analyzed using WinMDI 2.9 free software (BD).

**Caspase 3 activity assay**

The caspase activity was assessed by the FITC rabbit anti-active caspase 3 (BD Pharmingen). The cells were treated with Cu E for 24 h. Caspase 3 activation level was then measured by the flow cytometry (FACSCalibur). Data was analyzed using WinMDI 2.9 free software (BD).
Mitochondrial membrane potential

The cell lines were first seeded in 6-well plates (Orange Scientific). Following treatment with Cu E for four hours, JC-1 (25 μM) was added to the culture medium (500 μl/well) and incubated (37 °C, 20 min) for mitochondrial staining. After washing twice with warm PBS, the cells were fixed using 2% paraformaldehyde. For JC-1, quantification of fluorescence by flow cytometry (BD FACS calibur) and mitochondria containing red JC-1 aggregates in healthy cells were detectable in the FL-2 channel, and green JC-1 monomers in apoptotic cells were detectable in the FL-1 channel.

Cell cycle analysis

For cell cycle analysis we used the fluorescent nucleic acid dye propidium iodide (PI) to identify the proportion of cells in each of the three interphase stages of the cell cycle. The cells were treated with Cu E for 24 h, and then harvested and fixed in 1 ml cold 75% ethanol for overnight at −20 °C. DNA was stained in PI/RNaseA solution and the DNA content was detected using flow cytometry. Data was analyzed using WinMDI 2.9 free software.

DRS expression

Confocal microscopy was performed as described previously.25 Briefly, the cells (2 x 10⁶ cells) were fixed on coverslips. After treatment, they were incubated with mouse anti-DRS-phycocerythrin antibody (sc-166624 PE) (Santa Cruz BioTechnology, Dallas, TX, USA) for 30 min, and then washed with PBS. The cells were mounted onto microscope slides using mounting medium containing DAPI. The cells were first seeded in 6-well plates (Orange Scientific). Following treatment and anti-DRS-PE antibody incubated (37 °C, 20 min) for protein staining. After washing twice with PBS, the cells were fixed using 2% paraformaldehyde. For DRS, quantification by flow cytometry (BD FACS calibur).

Western blot assay

A total of 50–100 μg of proteins were separated by 10–12% SDS-PAGE, and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with blocking buffer (Odyddee, USA) overnight, and incubated with anti-β-actin (Sigma-Aldrich), anti-caspase 3 (sc-7148), anti-caspase 8 (sc-6134), anti-caspase 9 (sc-7885), anti-AIF (sc-9417) and DRS (sc-7192) antibodies for 1.5–2 h. The blots were washed and incubated with a second antibody (IRDye Li-COR, USA) or conjugated with horseradish peroxidase (HRP) at a 1/20,000 dilution for 30 min. The antigen was then visualized using a near infrared imaging system (Odyssey Li-COR, Lincoln, NE, USA) or chemiluminescence detection kit (ECL; Amersham Corp., Arlington Heights, IL, USA). The data was analyzed using Odyssey 2.1 software.

RT-PCR

A reverse transcriptase system (Promega, Southampton, UK) was used to synthesize cDNA from 1 μg of total RNA. Between 2 and 4 μl of cDNA were used for PCR analysis. PCR (50 μl) reactions were performed using 100 ng of each primer and 1 unit of Dyndyme II (Flowgen, Lichfield, UK). Thermal cycling was conducted for 35 cycles at the following temperature/durations: 96 °C for 10 s, 66 °C for 30 s, and 72 °C for 1 min using a Progene thermal cycler (Cambridge, UK). A final extension of 72 °C was performed for 10 min at the end of 35 cycles. The primers used for amplification of the target genes were checked against all other gene sequences for specificity. PCR reactions were analyzed on 1.5% agarose/TAE minigels and stained using 0.5 μg/ml ethidium bromide. Gels were visualized using an Apligene UV CCD camera system.

Real-time PCR

Real-time PCR was conducted using SYBR Green PCR Master Mix according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed using approximately 200 ng of SYBR Green PCR Master Mix in an ABI 7300 system (Applied Biosystems, Foster City, CA, USA). PCR conditions were 95 °C for 120 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. Sample cells from three plates were run in duplicate, using the threshold suggested by the software for the instrument to calculate Ct. To normalize readings, we used Ct values from 18s as internal controls for each run, obtaining a delta Ct value for each gene. (AIF: GATTGCAG CAGGGATCTCCAAAGA, R: GATTGACTCTCGGCAAATCTCCTC; Caspase 3 F: GGAACGGGAATCTGGACTGCG G: GCATCGACATGTAACAGC; Caspase 8 F: AAGACGGGTGACTCTTGAGGAAGA, R: TCAAGGACTCTCTCAAGGGGCTG; Caspase 9 F: TCTCCAGGTTGTGGCTTCTGG, R: CCTTCATTAAAGCAACGATT; BAX F: TCGAGGTACGGGCAAACAAAGAAG, R: TGTTGCCACCGGCGCCTACCATC; BCL-2 FGGCCACCTTCCTGAATGACCC; B: AACCAGGGTGAGCGGCTCT; and GAPDH F: GTCTCCCTGACTCCAAACGG; G: AACCACCCTGTTGCGGTAGCCAA).

Statistical analysis

All data were reported as the mean (±S.E.M.) of at least three separate experiments. A t-test or one-way ANOVA with post hoc test was employed for statistical analysis, with significant differences determined as P < 0.05.

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COMPETING INTERESTS
The authors declare no conflict of interest.

REFERENCES
1 Wu HH, Liu YF, Yang SF, Lin WL, Chen SC, Han CP et al. Association of single-nucleotide polymorphisms of high-mobility group box 1 with susceptibility and clinicopathological characteristics of uterine cervical neoplasia in Taiwanese women. Tumour Biol 2016; 37: 15813–15823.
2 Gaffney D, Small B, Kitchener H, Young Ryu S, Viswanathan A, Trimble T et al. Cervix Cancer Research Network (CCRN): improving access to cervix cancer trials on a global scale. Int J Gynecol Cancer 2016; 26: 1690–1693.
3 Yang A, Farmer E, Wu TC, Hung CF. Perspectives for therapeutic HPV vaccine development. J Biomed Sci 2016; 23: 75.
4 Tomait V. Functional roles of E6 and E7 oncoproteins in HPV-induced malignancies at diverse anatomical sites. Cancers 2016; 8: e95.
5 Wang HL, Lu X, Yang X, Xu N. Association of MBL2 exon1 polymorphisms with high-risk human papillomavirus infection and cervical cancers: a meta-analysis. Arch Gynecol Obstet 2016; 294: 1109–1116.
6 Cheng YT, Yang CC, Shyur LF. Phytomedicine-modulating oxidative stress and the tumor microenvironment for cancer therapy. Pharmacol Res 2016; 114: 128–143.
7 Amaral L, Spengler G, Molnar J. Identification of important compounds isolated from natural sources that have activity against multidrug-resistant cancer cell lines: effects on proliferation, apoptotic mechanism and the efflux pump responsible for multi-resistance phenotype. Anticancer Res 2016; 36: 5665–5672.
8 Hung CM, Chang CC, Lin CW, Chen CC, Hsu YC. GADD45y induces G2/M arrest in human pharynx and nasopharyngeal carcinoma cells by cucurbitacin E. Sci Rep 2014; 4: 6454.
9 Recio MC, Andujar I, Rios JL. Anti-inflammatory agents from plants: progress and potential. Curr Med Chem 2012; 19: 2088–2103.
10 Chen X, Bao J, Guo J, Ding Q, Lu J, Huang M et al. Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. Anticancer Drugs 2012; 23: 777–787.
11 Dinan L, Harmatha J, Lafont R. Chromatographic procedures for the isolation of plant steroids. J Chromatogr A 2001; 935: 105–123.
12 Attard E, Martinoli MG. Cucurbitacin E, an experimental lead triterpenoid with anticancer, immunomodulatory and novel effects against degenerative diseases. A mini-review. Curr Top Med Chem 2015; 15: 1708–1713.
13 Jevtić B, Djedović N, Stanisavljević S, Despotović J, Miljković D, Timotijević G. Cucurbitacin E potently modulates the activity of eucalyptoligenic cells. J Agric Food Chem 2016; 64: 4900–4907.
14 Duncan KL, Duncan MD, Alley MC, Saussville EA. Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. Biochem Pharmacol 1996; 52: 1553–1560.
15 Hsu YC, Huang TY, Chen MJ. Therapeutic ROS targeting of GADD45y in the induction of G2/M arrest in primary human colorectal cancer cell lines by cucurbitacin E. Cell Death Dis 2014; 5: e1198.
16 Habib L, Jrai A, Khreich N, Fessi H, Charcosset C, Greige-Gerges H. Morphological and physicochemical characterization of liposomes loading cucurbitacin E, an anti-proliferative natural tetracyclic triterpene. Chem Phys Lipids 2014; 177: 64–70.
17 Hung CM, Chang CC, Lin CW, Ko SY, Hsu YC. Cucurbitacin E as inducer of cell death and apoptosis in human oral squamous cell carcinoma cell line SAS. Int J Mol Sci 2013; 14: 17147–17156.
18 Naour GE, Tawadros F, Farooqi AA, Qureshi MZ, Tabassum S, Buchsbaum DJ et al. Role of nanotechnology and gene delivery systems in TRAIL-based therapies. Ecancermedicalscience 2016; 10: 660.
19 Mert U, Sanlioglu AD. Intracellular localization of DRS and related regulatory pathways as a mechanism of resistance to TRAIL in cancer. Cell Mol Life Sci 2016.
20 Kiraz Y, Adan A, Karal Yandim M, Baran Y. Major apoptotic mechanisms and genes involved in apoptosis. Tumour Biol 2016; 37: 8471–8486.
21 Gyurkovska V, Ivanovska N. Distinct roles of TNF-related apoptosis-inducing ligand (TRAIL) in viral and bacterial infections: from pathogenesis to pathogen clearance. Inflamm Res 2016; 65: 427–437.
22 Chen MJ, Tang WY, Hsu CW, Tsai YT, Wu JF, Lin CW et al. Apoptosis induction in primary human colorectal cancer cell lines and retarded tumor growth in SCID mice by sulforaphane. Evid Based Complement Alternat Med 2012; 2012: 415231.