Proteomic approaches to study epigallocatechin gallate-provoked apoptosis of TSGH-8301 human urinary bladder carcinoma cells: Roles of AKT and heat shock protein 27-modulated intrinsic apoptotic pathways

NIAN-GU CHEN¹, CHI-CHENG LU², YU-HSIN LIN³, WU-CHUNG SHEN⁴,⁷, CHENG-HUNG LAI¹, YUNG-JEN HO⁵,⁷, JING-GUNG CHUNG³, TSAI-HSIU LIN⁸, YUNG-CHANG LIN¹ and JAI-SING YANG⁶,⁹

Departments of ¹Veterinary Medicine, and ²Life Sciences, National Chung Hsing University, Taichung 402; Departments of ³Biological Science and Technology, ⁴Radiology, ⁵Biomedical Imaging and Radiological Science, ⁶Pharmacology, ⁷Radiology, and ⁸Laboratory Medicine, China Medical University Hospital, Taichung 404; ⁹Department of Cosmetic Science, Providence University, Taichung 433, Taiwan, R.O.C.

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Abstract. Epigallocatechin-3-gallate (EGCG), a polyphenol constituent present in green tea, has been shown to inhibit the growth of cancer cells in vitro and in vivo. However, studies regarding human bladder carcinoma cells are limited and not well investigated. Hence, our study focused on the evaluation of EGCG-triggered apoptosis in TSGH-8301 human urinary bladder carcinoma cells in vitro and in vivo as well as its related molecular mechanisms. In an in vivo study, EGCG inhibited xenograft tumor size of TSGH-8301 cells in a nude mouse model. Based on an in vitro study, EGCG resulted in morphological changes and increased growth inhibition in a dose- and time-dependent manner in TSGH-8301 cells. Furthermore, sub-G1 populations were shown and caspase-9 and -3 activities were stimulated in EGCG-treated TSGH-8301 cells. Moreover, a caspase-9 inhibitor (Z-LEHD-FMK) and a caspase-3 inhibitor (Z-DEVD-FMK) were able to reduce EGCG-stimulated caspase-9 and -3 activities, respectively. Loss of mitochondrial membrane potential (∆Ψm) resulted in an increase of protein levels of cytochrome c, Apaf-1, caspase-9 and -3 in TSGH-8301 cells following exposure to EGCG. Proteomic analysis revealed that EGCG affected the expression levels of various proteins, including HSP27, porin, tropomyosin 3 isoform 2, prohibitin and keratin 5, 14, 17 in TSGH-8301 cells. EGCG also suppressed AKT kinase activity and protein levels and also altered the expression levels of Bcl-2 family-related proteins such as Bcl-2, Bax, BAD and p-BAD. Based on the above findings, this study suggests that EGCG-provoked apoptotic death in TSGH-8301 cells is mediated through targeting AKT and HSP27 and modulating p-BAD, leading to activation of the intrinsic apoptotic pathway.

Introduction

Bladder cancer is one of the most common types of malignancies in human beings (1). Approximately 3.3 of 100 thousand people die from bladder cancer, and it is the 14th most common malignancy in Taiwan based on the report from the Department of Health, R.O.C. (Taiwan) in 2009 (2). In pathological analysis, >90% of bladder cancers are transitional cell carcinomas (TCC) (3). For the clinical therapy of bladder cancer patients, surgery, radiotherapy and chemotherapy may be used (4–6). However, these current therapies modalities are insufficient because the median survival time is about one year and it is unsatisfactory since distant metastasis may occur (7). Therefore, discovering a new more effective anti-bladder cancer agent is necessary.

Polyphenols are a group of chemical substances in human diets (8). The major sources of dietary polyphenol are fruits, vegetables and green tea (9,10). Many studies demonstrate that green tea can prevent chronic diseases and cancer in human bodies (11,12). The most important polyphenols in green tea include epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (13–15). EGCG has been reported to have biological functions (16–19) including anti-cancer activity (20). In vitro and in vivo studies have indicated that EGCG has chemopreventive and chemotherapeutic potential for many types of cancer cell lines (21–25). EGCG is thought to repress the growth of cancer cells
directly through cell-cycle arrest (26), induction of apoptosis (22,25), inhibition of metastasis (27), anti-angiogenesis (28) and activation of the immune function (29). These findings suggest that EGCG is an attractive potential agent for chemotherapy.

Several studies have demonstrated that induction of apoptosis in cancer cells could be a potential target for cancer treatment (10,13). Apoptosis is a regulated mechanism leading cells to death through the extrinsic and the intrinsic pathways. The extrinsic pathway is triggered by Fas ligand (FasL), tumor necrosis factor (TNF) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binding to their respective receptors such as Fas, TNF receptor (TNFR), death receptor 4 (DR4; TRAIL-R1) and DR5 (TRAIL-R2) (30). Interaction of specific ligands with their receptors leads to activation of Fas-associated death domain (FADD) and caspases-8 and -3 that is finally responsible for apoptosis (31). The intrinsic pathway is triggered by various stimuli such as DNA damage and chemotherapy agents. The intrinsic pathway involves disruption of the mitochondrial membrane environment and release of cytochrome c, Apaf-1, AIF, Endo G and procaspase-9 into the cytosol following activation of caspases-9 and -3 (32). Accumulating evidence indicates that heat shock protein 27 (HSP27) can regulate apoptosis through regulation of caspase activation (33,34). HSP27 is induced by stress and its function includes protection from heat shock, hypertonic stress and oxidative stress (33). Recent studies have shown that HSP27 has been closely associated with AKT activity which contributes to inhibition of cell apoptosis (34).

A study of Rieger-Christ et al reported that EGCG exhibited growth inhibitory activity in human bladder carcinoma cell lines including the J82, UM-UC-3, EJ, KK47, T24 and TCCSUP cells (35). Also, Qin et al demonstrated that EGCG promoted apoptosis of T24 human bladder cancer cells through modulation of the PI3K/AKT pathway and Bcl-2 family proteins (36). However, the mechanism by which EGCG affects human urinary bladder carcinoma cells specific for Taiwanese patients has not yet been clarified. In the present study, we focused on the anti-cancer effects and molecular mechanisms of EGCG in the human bladder cancer TSGH-8301 cells, a cell line derived from Taiwanese patients with urinary transitional cell carcinoma, in vivo and in vitro. We demonstrated that EGCG-induced apoptosis was carried out through AKT and the HSP27-modulated intrinsic apoptotic cascade pathways in TSGH-8301 cells.

Materials and methods

Chemicals and reagents. Epigallocatechin gallate (EGCG), dimethyl sulfoxide (DMSO), propidium iodide (PI), Triton X-100 and Trypan blue were obtained from Sigma-Aldrich Corp. (St. Louis, MO). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA). Caspase-9 and -3 activity colorimetric assay kits, and a caspase-9 inhibitor (Z-LEHD-FMK) and a caspase-3 inhibitor (Z-DEVD-FMK) were obtained from R&D Systems Inc. (Minneapolis, MN). The following primary antibodies were obtained: anti-caspase-9 and -3 (Cell Signaling Technology, Beverly, MA); anti-HSP27, cytochrome c, Apaf-1, p-AKT, AKT, Bcl-2, Bax, BAD, p-BAD, actin and horseradish peroxidase (HRP)-linked goat anti-mouse IgG and goat anti-rabbit IgG, (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell culture. The human urinary bladder carcinoma cell line (TSGH-8301) specific for patients in Taiwan, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm2 tissue culture flasks with RPMI-1640 medium with 2 mM L-glutamine containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37°C under a humidified 5% CO2 atmosphere (37).

Mouse xenograft model in vivo. The BALB/c nu/nu athymic mice (5-week-old) were purchased (National Laboratory Animal Center, Taipei, Taiwan) and randomly divided into 4 groups of 10 mice each. At 6 weeks of age, mice were subcutaneously injected with TSGH-8301 cells (1x107) in Matrigel (BD Biosciences, San Jose, CA, USA) into the flanks of mice. After the tumor volume reached 200 mm3, mice were gavaged with 100 µl PBS (control) or 12.5, 25 and 50 mg/kg/day EGCG in 100 µl PBS in each group. Tumor volume was monitored weekly based on the following formula: tumor volume (mm3) = LxW2/2 (L: length and W: width). At the end of 6 weeks of gavage treatment, mice were euthanized via CO2 asphyxiation. Cancer tissues from each animal were removed, measured and weighed individually as previously described (38,39). All animal studies were conducted according to institutional guidelines (Affidavit of Approval of Animal Use Protocol) approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

Cell morphology and viability determinations. TSGH-8301 cells (~2.5x105 cells/well) grown in 24-well plates were treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h. Then cells were examined and treated with EGCG at 0 and 75 µM and incubated at 37°C, 5% CO2 and 95% air for 72 h.

DNA content and analysis of the sub-G1 population by flow cytometry. Approximately 2.5x106 cells per well were seeded in 24-well plates and exposed to 75 µM of EGCG for 24, 48 and 72 h. Cells were divided into three independent experiments. The cell survival was shown as % of control (42,43).

Caspase-3/9 activities and their specific inhibitors. The activities of caspase-3 and -9 were measured by using caspase...
colorimetric assay kits according to the manufacturer's protocol (R&D Systems Inc.). Briefly, cells (5×10^6 cells) were seeded in 6-well plates and exposed to 75 µM of EGCG for 0, 12, 24, 48 and 72 h or control-treated before pretreatment without and with a caspase-9 inhibitor (Z-LEHD-FMK) or a caspase-3 inhibitor (Z-DEVD-FMK), respectively. Cells were harvested and lysed in 50 ml lysis buffer. The supernatant containing the protein was incubated with the caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA), respectively in reaction buffer. All samples were incubated in 96-well flat-bottom plates at 37°C for 1 h. The amounts of released pNA were measured at a wavelength of 405 nm with ELISA reader (13,42).

**Assay for mitochondrial membrane potential (ΔΨm).** Cells at a density of 2.5×10^6 cells were plated onto 24-well plates and exposed to 75 µM of EGCG for various periods of time (0, 6, 12 and 24 h). Cells were then harvested, washed, and re-suspended in 500 µl PBS with 50 mMol/l of 3,3'-diethoxydiamobenzidine iodide (DioC6, Invitrogen) at 37°C for 30 min to determine the level of ΔΨm. Cells were immediately measured by flow cytometry as previously described (45).

**Western blotting analysis.** TSGH-8301 cells (~1×10^7) were treated with 75 µM of EGCG for 0, 24 and 48 h as well as exposed to various concentrations of EGCG (0, 25, 50, 75 and 100 µM) for 6 h of incubation. Cells were collected by centrifugation and the total proteins were collected by using the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea). Equal amounts of protein lysate after determining the concentrations by the BCA assay (Pierce, Rockford, IL, USA) were run on a 10% SDS-PAGE gel and electrophoretically transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA) and probed with HSP27, cytochrome c, Apaf-1, caspase-9, caspase-3, p-AKT, AKT, Bel-2, Bax, BAD, and p-BAD plus actin as an internal control. Bands were visualized via chemiluminescence (ECL detection kit, Millipore) using HRP-conjugated secondary antibodies. The image is the outcome of protein as described elsewhere (46-48).

**AKT kinase assay.** Instructions in the non-radioactive AKT kinase assay kit (Cell Signaling Technology) were followed to determine the in vitro AKT kinase activity in EGCG-treated TSGH-8301 cells. Briefly, TSGH-8301 cells were grown up to 70% confluency in a 10-cm culture dish, and then treated for 6 h in the presence or absence of EGCG at 25, 50, 75 or 100 µM. Cells were harvested, washed with PBS and lysed in 1X cell lysis buffer. Immobilized AKT primary antibody bead slurry (20 µl) was added to 200 µl of whole-cell extract (200 µg of protein) overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer and twice with AKT kinase buffer. Kinase assays were performed in immuno-complexes (pellets) for 30 min at 30°C under continuous agitation in kinase buffer containing 200 µM ATP, 1 µg of glycerogen synthase kinase-3 (GSK-3) of fusion protein. The kinase reaction was terminated by adding 20 µl of 3X SDS sample buffer. The supernatant was saved for actin detection by immunoblotting to confirm that the same amount of whole cell extract was used and subjected to the monoclonal anti-AKT antibody. The protein level was quantified using the NIH ImageJ software and the activity was expressed as % of control. Each experiment was performed in duplicate, and the assays were repeated three times.

**Proteomic analysis.** At a density of 1×10^7 TSGH-8301 cells in 75 cm²-flask were incubated with or without 75 µM of EGCG for 6 h and then harvested for preparation as previously described (49). In the first dimension, isoelectric focusing (IEF) was performed with commercially available preformed immobilized pH gradients (IPGs) (linear pH gradient 3-10, 13 cm) by using an IPGphore IEF System (GE Healthcare Life Sciences, Piscataway, NJ, USA) with a three-phase program as previously described (49). Before second dimensional electrophoresis, the IPG gel strips were incubated at room temperature for 15 min and the gels were then submitted to a second dimension run on a Hoefer SE 600 (GE Healthcare Life Sciences) on running 12% SDS-PAGE gels (16x15 cm), without stacking gels. The 2-D polyacrylamide gels were stained with Coomassie Brilliant Blue (CBB) to assign the protein pl and MW, respectively. Data were considered significant when alterations were observed in at least two independent experiments (over five-fold up- or down-regulation) and the protein spots were consistently altered. Protein spots were quantified using the ImageMaster 2D Elite software (GE Healthcare Biosciences) as previously described (49). The procedures were modified as described by Kaji et al (50). Protein spots excised from the CBB-stained gel were destained and dried completely. The dried gels were rehydrated on ice for 45 min with a digestion buffer as previously described (49). Proteins were further digested at 37°C for 15 h after removing excess solution. The resultant peptides were extracted, desalted and concentrated using in-tip reverse phase resin as previously described (49). Peptides were isolated from each gel slice after in-gel digestion, desalted, and concentrated and peptide mixtures were eluted, applied to the sample target and air dried as previously described (49) and then the sample was analyzed in a nano-LC-MS/MS system. This analysis was performed on an integrated nano-LC-MS/MS system comprised of an LC Packings NanoLC system with an autosampler, and a QSTAR XL Q-Tof mass spectrometer (Applied Biosystems) fitted with nano-LC sprayer. Samples were first trapped and desalted on a LC Packings PepMap C18 µ-Precolumn Cartridge after the peptides were eluted off from the precolumn and separated on an analytical LC Packings PepMap C18 column connected inline to the mass spectrometer as previously described (49,51). The MS data were searched against the world wide web (WWW) search programs MS-Fit provided by http://www.matrixscience.com/search_form_select.html with the NCBI database.

**Statistical analysis.** Differences between untreated controls and treated-groups were calculated by one-way analysis of variance (ANOVA) followed by the Dunnett’s test. Results are presented as mean ± standard deviation (SD) and are representative of three independent experiments. A p-value <0.05 was considered significant.

**Results**

**Anti-tumor activity in vivo.** We investigated the effects of EGCG on in vivo transplantation cancer growth for 42 days in a xenograft model. EGCG inhibited TSGH-8301 tumor growth...
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in nude mice. EGCG was orally administered once every day at three different concentrations (12.5, 25 and 50 mg/kg). EGCG significantly reduced the tumor volume (Fig. 1A) and weight (Fig. 1B), respectively, in comparison of the control and EGCG-treated groups and these effects were dose-dependent responses.

EGCG causes morphological changes and growth inhibition in TSGH-8301 cells. The effects of treatment without and with EGCG on the morphological influences are shown in Fig. 2A. Our results indicate that TSGH-8301 cells exhibited clumping surface blebs and morphological shrinkage and rounding after exposure to 75 µM for 72 h. We then determined the cell growth inhibition effects and cell viability of EGCG in TSGH-8301 cells after treatment with 0, 25, 50, 75 and 100 µM for 24, 48, 72 h exposure by using the MTT assay. As can be seen in Fig. 2B, the cell viability was significant decreased in a dose- and time-dependent manner in EGCG-treated TSGH-8301 cells. The half maximal inhibitory concentration (IC50) in TSGH-8301 cells for 48 h exposure is close to 75 µM which was used for further experiments and assays in this study.

EGCG induces apoptosis in TSGH-8301 cells. Cells were treated with 75 µM of EGCG for 0, 24 and 72 h, and the DNA content for cell cycle distribution was analyzed by flow cytometry. EGCG-triggered cytotoxicity in TSGH-8301 cells may be mediated through induction of apoptosis and regulation of its signaling cascades. Flow cytometric profiles demonstrated that EGCG at 75 µM is able to increase the sub-G1 group in treated TSGH-8301 cells after 48 and 72 h of treatment (Fig. 3A). In addition, the sub-G1 populations showed significant increases in EGCG-treated TSGH-8301 cells after 24, 48 and 72 h of incubation and these effects were time-dependent (Fig. 3B).
EGCG stimulates caspase-9 and -3 activities in TSGH-8301 cells. To investigate the EGCG-induced apoptotic effects in TSGH-8301 cells, cells were incubated with 75 µM EGCG for 12, 24, 48 and 72 h and the activities of caspase-9 and -3 were determined by caspase-9 and -3 colorimetric assay kits, respectively. Treatment of TSGH-8301 cells with EGCG caused an increase of caspase-9 and -3 activities after 48 and 72 h of treatment (Fig. 4). To confirm the mechanisms of EGCG-induced apoptosis, cells were pretreated with a caspase-9 inhibitor (Z-LEHD-FMK) and a caspase-3 inhibitor (Z-DEVD-FMK) for 1 h and exposed to 75 µM of EGCG for 12-72 h in TSGH-8301 cells. Results demonstrated that Z-LEHD-FMK and Z-DEVD-FMK are able to attenuate the EGCG-stimulated caspase-9 and -3 activities, respectively, in TSGH-8301 cells (Fig. 4). Therefore, our results suggest that caspase-9 and -3 activation-mediated mitochondrial dysfunctions may be involved in the EGCG-provoked apoptotic death in TSGH-8301 cells.

EGCG increases the loss of ΔΨm and its associated protein levels. We next investigated whether or not EGCG-induced apoptosis is based on the mitochondria-dependent intrinsic cascade pathway. Cells were harvested for examining the level of ΔΨm after exposure to EGCG for various intervals of time. EGCG increased the loss of ΔΨm in TSGH-8301 cells (Fig. 5A). Therefore, we further investigated whether EGCG induced apoptosis of TSGH-8301 cells through the mitochondrial and intrinsic signaling pathways. Cells were treated with 75 µM of EGCG for 24, 48 and 72 h before analysis of protein levels by Western blotting. EGCG increased the protein levels of cytochrome c, Apaf-1, caspase-9 and -3 (Fig. 5B) in TSGH-8301 cells. Hence, this study suggests that EGCG-induced apoptotic death of TSGH-8301 cells is mediated through the mitochondrial and intrinsic cascade pathways.

Proteomics analysis for EGCG-induced apoptosis in TSGH-8301 cells. To explore the upstream molecular mechanisms of EGCG-induced apoptosis, the treatment condition with EGCG for 6 h was selected for the proteomic analysis. A pair of representative 2-D gel images for whole cell proteins extracted from TSGH-8301 cells with EGCG treatments for 6 h and control cells is shown in Fig. 6. Proteins were separated in the 2-D gels in the ranges of MW 14-100 kDa and pI 3-10. Image analysis revealed that a number of protein spots were significantly altered in terms of volume intensity. Highlighted in circles are the locations where protein alterations were detected. Table I lists the spot number, protein name, accession number, molecular weights, pIs, and sequence coverage from the programs MS-Fit. These altered proteins include the significant down-regulation of HSP27, porin (mitochondrial protein), tropomyosin 3 isoform 2, prohibitin (mitochondrial protein) and up-regulation of keratin 5, 14 and 17. Our data suggest that HSP27 and mitochondria may be involved in the major target of EGCG cytotoxicity in TSGH-8301 cells.
Table I. Summary of protein-database search results.

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<th>Sample no.</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>pI</th>
<th>MW (Da)</th>
<th>Seq. coverage</th>
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<td>29015</td>
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<tr>
<td>3</td>
<td>Prohibitin</td>
<td>gi</td>
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<td>29786</td>
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<tr>
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EGCG alters HSP27, p-AKT, Bcl-2, Bax, BAD and p-BAD protein levels as well as reduces AKT kinase activity in TSGH-8301 cells. To explore the upstream molecular mechanisms of EGCG-induced apoptosis, we evaluated the effect of EGCG on the HSP27, p-AKT, Bcl-2, Bax, BAD and p-BAD protein levels and AKT kinase activity in cell system. Western blot analysis identified reduction of HSP27 and phospho-AKT as molecular determinants of EGCG-induced apoptosis in TSGH-8301 cells in a dose-dependent manner (Fig. 7A). Thus, these results suggest that EGCG-triggered apoptosis is involved in the decrease of the protein levels in HSP27 and p-AKT in TSGH-8301 cells. It has been reported that the pro-apoptotic protein BAD, a member of the Bcl-2 family, is rendered inactive when the phosphorylated serine/threonine protein kinase p-AKT converts p-BAD and BAD. To investigate the downstream effectors, we traced the phosphorylation status of BAD and also assessed the protein levels of Bcl-2 family, including Bcl-2, Bax and BAD in EGCG-treated TSGH-8301 cells with 0, 50, 75 and 100 µM for 24-h exposure.
and p-BAD rather than increased the protein levels of Bax and BAD proteins in TSGH-8301 cells (Fig. 7A). Based on these results, apoptosis of TSGH-8301 cells appears to be mediated through increasing the ratio of Bax/BAD/Bcl-2 level after EGCG exposure. Moreover, TSGH-8301 cells were harvested after treatment with 0, 25, 50, 75 and 100 µM of EGCG for 6 h to measure AKT activity. Our results reveal that EGCG down-regulated the AKT activity after 50-100 µM EGCG for 6 h of treatment and this inhibition was time-dependent (Fig. 7B). We suggest that these responses are involved in the suppression of AKT activity, which may be regulated by p-BAD. These results signify the involvement of the HSP27/AKT/BAD survival pathway in the EGCG-induced apoptotic death of TSGH-8301 cells.

Discussion

Many studies have reported that EGCG has growth inhibitory effects in human cancer cell lines (21-27,54,55), but few have addressed its inhibitory effects on Taiwan-specific human urinary bladder carcinoma cells. In this study, we examined the cytotoxicity of EGCG in TSGH-8301 cells in vitro and examined EGCG-affected TSGH-8301 cells in vivo. Our data indicate that EGCG inhibited xenograph tumor size of TSGH-8301 cells in a nude mouse model in vivo (Fig. 1). On the other hand, EGCG at 25-100 µM could have significant cytotoxicity effects on TSGH-8301 cells in vitro (Fig. 2). Our previous study revealed that EGCG exerts low cytotoxicity on normal cells such as Detroit 551 normal human cell line (13). Our data suggest that EGCG represents a promising candidate as an anti-urinary bladder carcinoma agent with low toxicity to normal cells.

In this study, we have shown that EGCG triggered morphological changes (Fig. 2) and reduced the cell viability in TSGH-8301 cells in a dose- and time-dependent manner. EGCG induced accumulation of the sub-G1 population (apoptosis, Fig. 3). Moreover, our results demonstrate that EGCG significantly promoted activities of caspase-9 (Fig. 4A) and caspase-3 (Fig. 4B) after 24-72 h treatment, but there was no significant effect in caspase-8 activity (data not shown). EGCG increased the protein levels of Bax and BAD, and inhibited the level of Bcl-2 (Fig. 7A) which led to the disruption of ΔΨm (Fig. 5A) and the release of cytochrome c, AIF and pro-caspase-9 (Fig. 5B). Furthermore, EGCG reduced the levels of porin (the voltage-dependent anion channel, regulating the mitochondrial response to certain cell death stimuli) and prohibitin (stabilization of mitochondrial function) by proteomic analysis (Fig. 6 and Table 1). EGCG increased caspase-9 and -3 activities (Fig. 4), and pretreatment with caspase-9,-3 inhibitors led to decreased caspase-9 and -3 activities, resulting in keratin cleavage (Figs. 4 and 6). Qin et al demonstrated that EGCG promotes apoptosis in T24 human bladder cancer cells through modulation of the PI3K/AKT pathway and Bcl-2 family proteins (36). It has been reported that AKT is involved in the survival signaling pathway by phosphorylating BAD (56,57). In the present study, EGCG inhibited the activity of AKT in TSGH-8301 cells (Fig. 7B), leading to that BAD was dephosphorylated and then induced apoptosis in EGCG-treated TSGH-301 cells. Our results suggest that the intrinsic pathway is the major pathway of EGCG-induced apoptosis in TSGH-8301 cells. Several studies have reported that EGCG induces cell apoptosis through the extrinsic apoptotic pathway in HepG2 and U937 cells (58,59). In our previously studies, we also demonstrated that EGCG-induced apoptosis is mainly mediated by induction of the extrinsic and intrinsic pathways in human adrenal cancer NCI-H295 cells (13). Of note, EGCG-provoked cell death occurs through various apoptotic signaling pathways in different types of tumor cell lines in vitro.

EGCG could be developed as a new class of chemopreventive or chemotherapeutic agent for cancer because it targets multiple pathways and resulted in inhibition of cancer cell growth (13,58-60). Recent studies suggest that EGCG may affect many important signaling pathways, including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), mitogen-activated protein kinase (MAPK), nuclear factor-xB (NF-xB), phosphatidylinositol 3-kinase (PI3K)/AKT and heat shock protein 27 (33,34,61). In this study, we initially demonstrated that EGCG significantly suppressed HSP27 protein level in TSGH-8301 cells by Western blotting and proteomic analyses (Figs. 6 and 7). In previous study, we have shown that HSP27 is associated with the regulation of F-actin assembly and is a known regulator of AKT (33,34). Konishi et al have shown that in COS-7 cells treated with H2O2, AKT is activated and the association of HSP27 with AKT increases concurrently with the enhancement of AKT activity (62). Natsume et al have reported that EGCG induced suppression of the AKT cascade in osteoblasts contributes to the modulation of osteoblastic cell function toward bone formation via specifically down-regulating HSP27 induction (63). This evidence suggests that HSP27 could regulate AKT activity. However, another report has demonstrated that HSP27 directly inhibits caspase-9 and -3 activation and reduces apoptosis forma-

Figure 8. Illustration of the proposed signaling pathways of the EGCG-triggered apoptotic death and its modulated upstream HSP27/AKT signal in human urinary bladder carcinoma TSGH-8301 cells.

...and p-BAD rather than increased the protein levels of Bax and BAD proteins in TSGH-8301 cells (Fig. 7A). Based on these results, apoptosis of TSGH-8301 cells appears to be mediated through increasing the ratio of Bax/BAD/Bcl-2 level after EGCG exposure. Moreover, TSGH-8301 cells were harvested after treatment with 0, 25, 50, 75 and 100 µM of EGCG for 6 h to measure AKT activity. Our results reveal that EGCG down-regulated the AKT activity after 50-100 µM EGCG for 6 h of treatment and this inhibition was time-dependent (Fig. 7B). We suggest that these responses are involved in the suppression of AKT activity, which may be regulated by p-BAD. These results signify the involvement of the HSP27/AKT/BAD survival pathway in the EGCG-induced apoptotic death of TSGH-8301 cells.

Discussion

Many studies have reported that EGCG has growth inhibitory effects in human cancer cell lines (21-27,54,55), but few have addressed its inhibitory effects on Taiwan-specific human urinary bladder carcinoma cells. In this study, we examined the cytotoxicity of EGCG in TSGH-8301 cells in vitro and examined EGCG-affected TSGH-8301 cells in vivo. Our data indicate that EGCG inhibited xenograph tumor size of TSGH-8301 cells in a nude mouse model in vivo (Fig. 1). On the other hand, EGCG at 25-100 µM could have significant cytotoxicity effects on TSGH-8301 cells in vitro (Fig. 2). Our previous study revealed that EGCG exerts low cytotoxicity on normal cells such as Detroit 551 normal human cell line (13). Our data suggest that EGCG represents a promising candidate as an anti-urinary bladder carcinoma agent with low toxicity to normal cells.

In this study, we have shown that EGCG triggered morphological changes (Fig. 2) and reduced the cell viability in TSGH-8301 cells in a dose- and time-dependent manner. EGCG induced accumulation of the sub-G1 population (apoptosis, Fig. 3). Moreover, our results demonstrate that EGCG significantly promoted activities of caspase-9 (Fig. 4A) and caspase-3 (Fig. 4B) after 24-72 h treatment, but there was no significant effect in caspase-8 activity (data not shown). EGCG increased the protein levels of Bax and BAD, and inhibited the level of Bcl-2 (Fig. 7A) which led to the disruption of ΔΨm (Fig. 5A) and the release of cytochrome c, AIF and pro-caspase-9 (Fig. 5B). Furthermore, EGCG reduced the levels of porin (the voltage-dependent anion channel, regulating the mitochondrial response to certain cell death stimuli) and prohibitin (stabilization of mitochondrial function) by proteomic analysis (Fig. 6 and Table 1). EGCG increased caspase-9 and -3 activities (Fig. 4), and pretreatment with caspase-9,-3 inhibitors led to decreased caspase-9 and -3 activities, resulting in keratin cleavage (Figs. 4 and 6). Qin et al demonstrated that EGCG promotes apoptosis in T24 human bladder cancer cells through modulation of the PI3K/AKT pathway and Bcl-2 family proteins (36). It has been reported that AKT is involved in the survival signaling pathway by phosphorylating BAD (56,57). In the present study, EGCG inhibited the activity of AKT in TSGH-8301 cells (Fig. 7B), leading to that BAD was dephosphorylated and then induced apoptosis in EGCG-treated TSGH-301 cells. Our results suggest that the intrinsic pathway is the major pathway of EGCG-induced apoptosis in TSGH-8301 cells. Several studies have reported that EGCG induces cell apoptosis through the extrinsic apoptotic pathway in HepG2 and U937 cells (58,59). In our previously studies, we also demonstrated that EGCG-induced apoptosis is mainly mediated by induction of the extrinsic and intrinsic pathways in human adrenal cancer NCI-H295 cells (13). Of note, EGCG-provoked cell death occurs through various apoptotic signaling pathways in different types of tumor cell lines in vitro.

EGCG could be developed as a new class of chemopreventive or chemotherapeutic agent for cancer because it targets multiple pathways and resulted in inhibition of cancer cell growth (13,58-60). Recent studies suggest that EGCG may affect many important signaling pathways, including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), vascular endothelial growth factor receptor (VEGFR), mitogen-activated protein kinase (MAPK), nuclear factor-xB (NF-xB), phosphatidylinositol 3-kinase (PI3K)/AKT and heat shock protein 27 (33,34,61). In this study, we initially demonstrated that EGCG significantly suppressed HSP27 protein level in TSGH-8301 cells by Western blotting and proteomic analyses (Figs. 6 and 7). In previous study, we have shown that HSP27 is associated with the regulation of F-actin assembly and is a known regulator of AKT (33,34). Konishi et al have shown that in COS-7 cells treated with H2O2, AKT is activated and the association of HSP27 with AKT increases concurrently with the enhancement of AKT activity (62). Natsume et al have reported that EGCG induced suppression of the AKT cascade in osteoblasts contributes to the modulation of osteoblastic cell function toward bone formation via specifically down-regulating HSP27 induction (63). This evidence suggests that HSP27 could regulate AKT activity. However, another report has demonstrated that HSP27 directly inhibits caspase-9 and -3 activation and reduces apoptosis formation (64). Our results showed that when expressed to EGCG in TSGH-8301 cells, HSP27, markedly decreased protein levels (Fig. 7A), induced the leakage of cytochrome c, Apaf-1 and...
pro-caspase-9 (Fig. 2A), exhibited caspase-9 and -3 activation (Fig. 4), and significantly improved cell apoptosis (Figs. 2A and 3). Our results suggest that HSP27 could either directly or indirectly induce apoptosis in EGCG-treated TSGH-8301 cells. The direct effect is that HSP27 inhibits caspase-9 and -3 activation and reduces apoptosisome formation. The indirect effect is that HSP27 regulates BAD phosphorylation by altering the AKT kinases activity.

In conclusion, in vitro results indicate that the EGCG effects in the TSGH-8301 xenograft tumor model of human urinary bladder carcinoma was due to its anti-tumor activity. The proposed model of EGCG mechanism of action for apoptosis in TSGH-8301 cells is shown in Fig. 8. EGCG induced apoptosis in TSGH-8301 cells is mediated through the HSP27 and AKT pathways, which involve BAD phosphorylation and activation of caspase-9 and -3. Proteomic results and mitochondrial functional studies confirm that the intrinsic pathway is the major apoptotic pathway of EGCG in TSGH-8301 cells. EGCG deserves further investigation in preclinical studies or in a clinical trial as a potential anti-urinary bladder carcinoma agent.

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