Molecular Mechanisms of (-)-Gossypol-induced Apoptosis in Human Prostate Cancer Cells

YI-WEN HUANG[1], LI-SHU WANG[1], HSIANG-LIN CHANG[1], WEIPING YE[1],
MICHAEL K. DOWD[3], PETER J. WAN[3] and YOUNG C. LIN[1,2]

[1]Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine and
[2]OSU Comprehensive Cancer Center, The Ohio State University, Columbus, OH;
[3]USDA, Southern Regional Research Center, New Orleans, LA, U.S.A.

Abstract. *Background: Gossypol, a natural compound present in cottonseeds, displays antiproliferative and pro-apoptotic effects against various cancer cells. The (-)-gossypol enantiomer is a more potent inhibitor of cancer cell growth. Here, the molecular mechanisms of apoptosis induced by (-)-gossypol were studied in human prostate cancer cells. Materials and Methods: After the prostate cancer cell DU-145 had been treated with (-)-gossypol, the trypan blue exclusion assay and DNA fragment end-labeling assay were used to stain the dead cells and to detect DNA laddering, respectively. The effects of (-)-gossypol on the expression of apoptotic-regulated gene markers in both death receptor- and mitochondria-mediated apoptotic pathways, such as the Bcl-2 family and caspase, etc., were detected by RT-PCR and Western blot analysis. To further investigate the apoptotic pathways induced by (-)-gossypol, different caspase inhibitors were used to block caspase activities and cell viability was detected by the CellTiter 96™ AQueous assay in DU-145 cells. Results: At a 5-10 μM dose-level, (-)-gossypol significantly enhanced apoptosis measured by DNA fragmentation. (-)-Gossypol caused apoptosis in DU-145 cells through the down-regulation of Bcl-2 and Bcl-xL and the up-regulation of Bax at the mRNA and protein levels. (-)-Gossypol also activated caspases-3, -8 and -9 and increased PARP [poly (ADP-ribose) polymerase] cleavage. Furthermore, (-)-gossypol-induced apoptosis might be due to an increase in CAD (caspase-activated deoxyribonuclease) proteins and a decrease in ICAD (inhibitor of CAD) proteins. By using caspase inhibitors, (-)-gossypol caused apoptosis via the caspase-dependent pathways.*

Correspondence to: Young C. Lin, DVM, Ph.D., Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, U.S.A. Tel: +1 614 292-9706, Fax: +1 614 292-7599, e-mail: lin.15@osu.edu

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Conclusion: Our results indicated that the apoptotic processes caused by (-)-gossypol are mediated by the regulation of the Bcl-2 and caspase families in human prostate cancer cells. Our data also suggested that (-)-gossypol may have chemotheraputic benefits for prostate cancer patients.

Prostate cancer is the most commonly diagnosed non-skin cancer in the United States. One in six American men will develop prostate cancer during the course of his lifetime. In 2005, over 230,000 men will be diagnosed with prostate cancer, and nearly 30,000 men are expected to die from it (1). Prostate cancer can be eliminated by surgery or radiation, if diagnosed at an early stage. However, each year, 70,000 men require additional treatment due to a recurrence of their prostate cancer (1).

Gossypol (*Gossypium L., Malvaceae*) is a naturally occurring polyphenolic pigment present in cottonseeds and in cotton plant by-products, such as cottonseed oil and cottonseed meal flour, that often are consumed by humans and food-producing animals. Previously, the vast majority of research on gossypol and cancer focused on the *in vitro* and *in vivo* antiproliferative activities of racemic gossypol, (±)-gossypol, in a variety of cancers including breast, ovary, cervix, uterus, adrenals, pancreas, colon and head and neck. Research has recently been focused on (-)-gossypol's pro-apoptotic activity.

Our laboratory demonstrated the antiproliferative effects of (±)-gossypol on human breast cancer cells (2, 3) and also reported that (±)-gossypol possessed anticancer activity in human (4, 5), canine (6, 7) and rat prostate cancer (8). The anticancer effects of (±)-gossypol have been attributed to its (-)-enantiomer, which is a more potent inhibitor of cancer cell growth (9, 10). Our previous results also demonstrated that (-)-gossypol was more effective at inhibiting the growth of adipose stromal cells (11) and human breast cancer cells (12).

Apoptosis is one of the major processes leading to cell death. The Bcl-2 family of proteins constitutes a critical

intracellular checkpoint of apoptosis (13). Bcl-2 was initially identified at the chromosomal breakpoint of t(14;18)-bearing human follicular B-cell lymphoma cells (13). Mammals possess an entire family of Bcl-2 proteins that include pro-apoptotic as well as anti-apoptotic members. The Bcl-2 family can be divided into three main subclasses: (I) the anti-apoptotic members: Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-w; (II) the multidomain pro-apoptotic members: Bax, Bak and Bok; and (III) the BH3-only pro-apoptotic members: Bid, Bad, Bim, Noxa and Puma (14).

Gossypol was found to bind with Bcl-2 and Bcl-xL in computer-assisted molecular modeling and fluorescence-polarization assays (15). Recently, (-)-gossypol was demonstrated to inhibit cell growth and to induce apoptosis in cancer cells engineered to overexpress Bcl-2 or Bcl-xL. Gossypol acted directly on the mitochondria to release cytochrome *c* (16). Moreover, (-)-gossypol enhanced the effect of radiation therapy of prostate cancer *in vitro* and *in vivo* without augmenting toxicity (17). In human colon carcinoma, ($\pm$)-gossypol induced apoptosis by triggering the down-regulation of the anti-apoptotic Bcl-2 members, Bcl-xL, Bag-1 and Mcl-1; the up-regulation of the pro-apoptotic Bcl-2 member: Bak; activation of caspases-3, -6, -7, -8 and -9; the up-regulation of Apaf-1; increasing the release of cytochrome *c* from the mitochondria; and the activation of both ICAD (also called DFF45) and PARP (18). Moreover, gossypol-mediated apoptosis was associated with increases of Fas (also called APO-1 or CD95 as a tumor necrosis factor receptor) and Fas ligand (FasL) in lung cancer cells (19). These findings suggested that gossypol induces apoptosis through both intrinsic and extrinsic pathways in several cancer models. However, little is known about the molecular mechanisms of gossypol-induced apoptosis in human prostate cancer cells.

In the present study, the molecular mechanisms of (-)-gossypol-involved cell apoptosis regulation in prostate cancer cells were investigated. Our results demonstrated that (-)-gossypol-induced cell apoptosis occured through the suppression of anti-apoptotic Bcl-2 members and extensive caspases (caspases-3, -8 and -9) and the activation of PARP. Moreover, (-)-gossypol induced cell apoptosis in a caspase-dependent manner. Finally, our observations suggested that the consumption of (-)-gossypol-containing cotton products (cottonseed oil and cottonseed meal flour) might be beneficial for prostate cancer patients, as well as for healthy individuals.

Materials and Methods

Cells and reagents. The human prostate cancer cell line DU-145 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1)

(DMEM/F12) without phenol red (Atlanta Biologicals, Norcross, GA, USA) supplemented with 5% fetal bovine serum (FBS) and antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (Invitrogen, Bethesda, MD, USA).

Antibodies against Bcl-2, Bcl-xL, Bax, Bad, caspases-3, -8 and -9 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against CAD, ICAD and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse IgG-HRP conjugates were from GE Healthcare (Piscataway, NJ, USA). Caspase inhibitors were purchased from Santa Cruz Biotechnology. (-)-Gossypol was provided by co-author Dr. Michael Dowd from the USDA, Southern Regional Research Center (New Orleans, LA, USA) and the (-)-gossypol preparation has been described in detail previously (20).

Cell viability assay. The effect of the caspase inhibitors on (-)-gossypol-suppressed cell viability was assessed by CellTiter 96T™ AQueous (Promega, Madison, WI, USA) assay in 96-well plates, according to the manufacturer's instructions. A total of 4,000 cells/well was seeded and cultured in 6-well plates in phenol red-free DMEM/F12 supplemented with 5% FBS overnight. The medium was switched to DMEM/F12 supplemented with Dextran-coated charcoal (DCC) (Dextran T-70, Pharmacia; activated charcoal, Sigma)-stripped FBS (5%). The cells were then pretreated with caspase inhibitors for 6 h prior to the addition of (-)-gossypol and thereafter in the culture throughout another 24 h. The cell viability was determined using a colorimetric method for the number of viable cells in culture in a multi-well plate format. At the end of the treatment, the cell viability was measured by adding a mix of 20 μl freshly combined MTS and PMS (the ratio of MTS:PMS at 20:1) solution to each well. The plates were then incubated for 30 min and the color density was measured as the optical density at 490 nm (OD 490 nm) using an ELISA plate reader.

Dead cell number. The effect of (-)-gossypol on the dead cell number was assessed by the trypan blue dye exclusion method. Prostate cancer cells were seeded separately at a density of 1×10^5 cells/well in 6-well plates for 24 h and were allowed to attach. The medium was replaced with fresh DMEM/F12 with 5% DCC-FBS. The cells were exposed to (-)-gossypol at the indicated concentrations in 5% DCC-FBS-supplemented medium, at 37°C in 5% CO_2 for 24 h. Adherent cells were detached by rapid trypsinization and the total cells including floating cells were collected and counted. An adequate volume of medium containing trypan blue was added. The cells were then counted using a hemacytometer. The cells stained with trypan blue appeared dark-blue and were considered dead. The percentage of apoptotic cells was calculated by: dead cells/total cells.

DNA fragment end-labeling assay. (-)-Gossypol-induced apoptosis in prostate cancer cells was detected by DNA laddering. The procedure was slightly modified from a previous article (21). Briefly, DU-145 cells were cultured in T75 flasks. After (-)-gossypol exposure, trypsinized and floating cells were collected by centrifugation. The cell pellets were resuspended in 1 ml Hanks' buffered salt solution (HBSS), transferred into 10 ml of ice-cold 70% ethanol and stored at –20°C for 24 h or longer. The fixed cells were centrifuged and the ethanol was completely removed. The cell

Table I. *Primer sequences and PCR amplification of apoptosis-related genes.*

Gene	Primer sequences	Anneal (°C)	MgCl$_2$ (mM)	Cycle number	Product size (bp)
Bax	5'-ATGGACGGGTCCGGGGAGCA-3'				
	5'-CCCAGTTGAAGTTGCCGTCA-3'	58	1.0	30	322
Bak	5'-ACGCTATGACTCAGAGTTCC-3'				
	5'-CTTCGTACCACAAACTGGCC-3'	63	3.0	30	360
Bcl-2	5'-GTGAACTGGGGGAGGATTGT-3'				
	5'-GGAGAAATCAAACAGAGGCC-3'	63	1.5	39	216
Bcl-xL	5'-CCCAGAAAGGATACAGCTGG-3'				
	5'-GCGATCCGACTCACCAATAC-3'	56	1.5	32	448
36B4	5'-AAACTGCTGCCTCATATCCG-3'				
	5'-TTGATGATAGAATGGGGTACTGAT G-3'	63	3.5	30	563

pellets were resuspended in 40 μl phosphate-citrate buffer, consisting of 192 parts of 0.2 M Na$_2$HPO$_4$ and 8 parts of 0.1 M citric acid (pH 7.8), and were incubated at room temperature for at least 30 min. The cells were centrifuged and the supernatants were transferred to new tubes. Three μl of 0.25% Nonidet NP-40 and 3 μl of RNAse (1 mg/ml) were added to the samples which were incubated for 30 min at 37°C. After incubation, 3 μl of proteinase K (1 mg/ml) were added to the samples, which were incubated for another 30 min at 37°C. The DNA was collected by centrifugation, separated on a 1.5% agarose gel, stained with ethidium bromide and digitally imaged under UV light.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). A total of 1x10^5 cells was seeded in each well of six-well plates with 2 ml of culture medium in the presence or absence of (-)-gossypol (1 to 5 μM). Total RNA was isolated in 1 ml of TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed as described previously (12). Briefly, 1 μg of total RNA from the cultured cells was reverse transcribed with 200 U M-MLV Reverse Transcriptase (Invitrogen), at 37°C for 50 min, in the presence of 1 μl of 10 mM dNTP mix (dATP, dCTP, dGTP and dTTP), 10 μl 5X first-strand buffer, 0.01 M DDT, 1 μl RNAase inhibitor (Invitrogen) and 1 μM random hexamers in a total volume of 50 μl. The reaction was terminated by heating to 95°C for 3 min.

The newly synthesized cDNAs were used as templates for PCR after adjusting the reagent concentrations to 1.0-3.5 mM MgCl$_2$, 2.5 μl 10X PCR buffer, 1U Platinum® Taq DNA polymerase (Invitrogen) and 0.24 μM primers. The reactions were incubated at 95°C for 5 min. Then, amplification was performed with each cycle consisting of denaturation at 95°C for 1 min, annealing at various temperatures for 1 min and extension at 72°C for 1 min, in an Eppendorf thermal cycler (Westbury, NY, USA). The primers and amplified conditions of apoptosis-related genes (Bak, Bax, Bcl-2 and Bcl-xL) are described in Table I. The final PCR products (10 μl) were mixed with 1 μl of 10X loading buffer, followed by separation on a 1.5% agarose gel containing ethidium bromide. The specific bands of mRNA expression were measured by densitometry ethidium bromide-stained PCR products.

Western blot analysis. After incubation with different concentrations of (-)-gossypol (0, 1, 2.5 or 5 μM) for 24 h, the DU-145 cells were washed with ice-cold PBS and then lysed in M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA) with protease inhibitor (Pierce). The cell lysates were separated by centrifugation at 15,000 xg at 4°C for 25 min. An equivalent amount of protein (50 μg) from each supernatant was boiled with the sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.004% bromophenol blue and 5% β-mercaptoethanol) for 5 min and resolved on a 4-15% SDS-polyacrylamide Ready Gel (Bio-Rad, Hercules, CA, USA). Following electrophoresis, the proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore) using a semi-dry transfer system (Bio-Rad) at room temperature. The transblotted membranes were washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) and were then blocked with PBST including 10% non-fat milk for 1 h. Subsequently, the membranes were incubated with the appropriate primary antibody in PBST containing 5% non-fat milk at 4°C overnight and all the primary antibodies diluted 1:1000. Following incubation in the primary antibody, the membranes were briefly rinsed and washed twice with PBST for a total of 20 min, followed by incubation in goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate (diluted 1:5000) for 1 h at room temperature. After incubation, the membranes were rinsed and washed. The immunoblots were enhanced by ECL Plus reagent (GE Healthcare) and visualized using the Fuji imaging system (Fujifilm Medical Systems U.S.A., Stamford, CT, USA).

Statistical analysis. The results of the cell viability assay and dead cell number are presented as one group by the mean±standard deviation (s.d.) for three or six replicate culture wells. The analysis was performed using the StatView® ANOVA unpaired *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Results

(-)-Gossypol-induced apoptosis and DNA fragmentation in DU-145 cells. To examine the apoptotic effect of (-)-gossypol on cell death and DNA fragmentation, the DU-145 cells were cultured and treated with either (-)-gossypol (1 to 20 μM) or the vehicle for 24 h. Dead cells were counted using trypan blue staining under a microscope. At the 10 and 20 μM dose levels, (-)-gossypol induced significant cell death (55 and 85%,
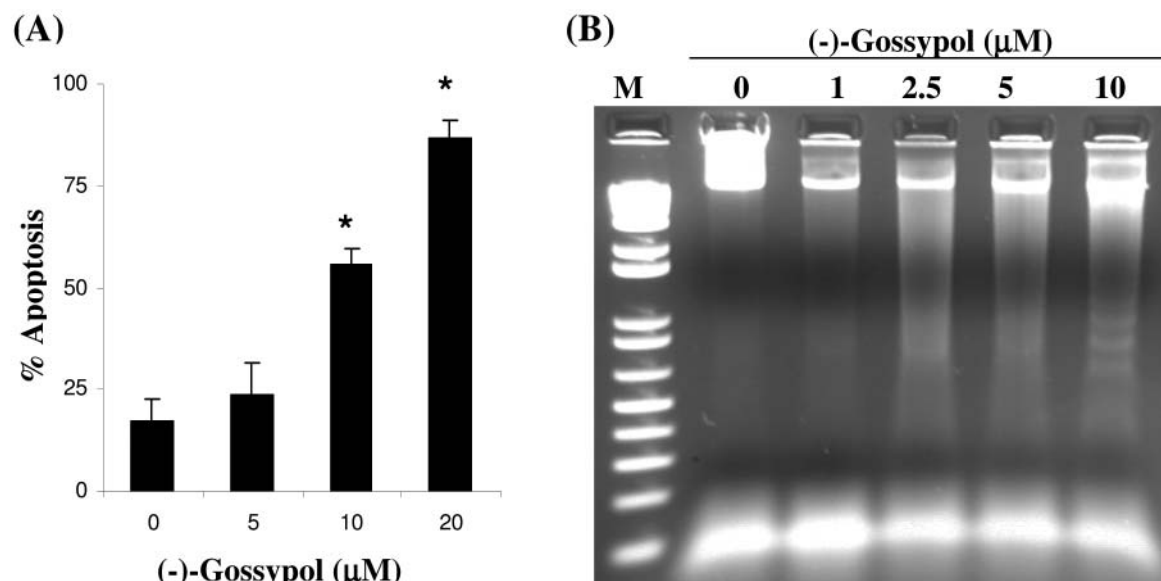
Figure 1. (-)-Gossypol-induced apoptosis and DNA fragmentation in DU-145 cells. DU-145 cells were treated with different concentrations of (-)-gossypol or the vehicle control for 24 h. (A) Dead cells were counted using a hemacytometer under trypan blue staining. Each bar represents the mean ± s.d. of four wells. The asterisks show significant differences from the control group (p<0.05). (B) After exposure to (-)-gossypol, the cells were harvested and DNA was extracted, as described in the Materials and Methods section. The DNA was separated on 1.5% agarose and digitally imaged after staining with ethidium bromide. M was marker.

respectively) (Figure 1A). Furthermore, (-)-gossypol induced DNA fragmentation (Figure 1B). A lack of DNA fragmentation at the 1 μM-treatment was observed, however, the presence of small fragments of DNA gradually increased between 5 and 10 μM concentrations of (-)-gossypol (Figure 1B). These data indicated that (-)-gossypol can induce cell apoptosis and DNA fragmentation.

(-)-Gossypol altered the mRNA and protein expressions of the Bcl-2 family in human prostate cancer cells. The Bcl-2 family is a central regulator of cell apoptosis. It was found that gossypol bound Bcl-2 and Bcl-xL and potently induced apoptosis in several cancer cell lines (15, 18). After observing the induction of apoptosis by (-)-gossypol, whether the Bcl-2 family is involved in (-)-gossypol-induced apoptosis was examined.

The DU-145 cells were exposed to (-)-gossypol (1 to 5 μM) or vehicle for 24 h. The expression levels of mRNA and proteins of the Bcl-2 family were measured by RT-PCR and Western blot analysis, respectively.

The data revealed that (-)-gossypol regulated both the mRNA (Figure 2A) and protein (Figure 2B) expressions of the Bcl-2 family members. For the prostate cancer cells treated with 1 and 2.5 μM (-)-gossypol, the levels of Bcl-2, Bcl-xL and Bax mRNA were not significantly altered. However, at 5 μM, (-)-gossypol dramatically decreased the Bcl-2 and Bcl-xL mRNA expressions, but increased Bax

mRNA expression in the DU-145 cells (Figure 2A). Regarding protein expression, (-)-gossypol significantly down-regulated the Bcl-2 and Bcl-xL levels, but significantly up-regulated Bax expression. Only a slight increase of Bad levels caused by (-)-gossypol was observed (Figure 2B). The alteration of protein expression occurred in a dose-dependent manner. Based on these observations, (-)-gossypol-induced prostate cancer cell apoptosis may be modulated by reducing the expression of the anti-apoptotic Bcl-2 family and by inducing the expression of the pro-apoptotic Bcl-2 family.

(-)-Gossypol activated caspases, PARP and CAD in human prostate cancer cells. In order to further investigate the effect of (-)-gossypol on the downstream molecular markers of apoptosis, capsase protein expression was examined by Western blot after treatment of the cells with (-)-gossypol. The caspases exist as inactive pro-enzymes that undergo proteolytic processing at conserved aspartic residues to produce a large and a small subunit. Two initiators of caspases-8 and -9 were examined and (-)-gossypol was found to reduce the amount of full-form caspase-9 and to increase the amount of cleaved caspase-9 at the 5 μM treatment level. In addition, (-)-gossypol decreased full-form of caspase-8 in a dose-dependent manner, although, no cleaved caspase-8 was observed, indicating that (-)-gossypol might induce caspase-8 degradation instead of activation (Figure 3).
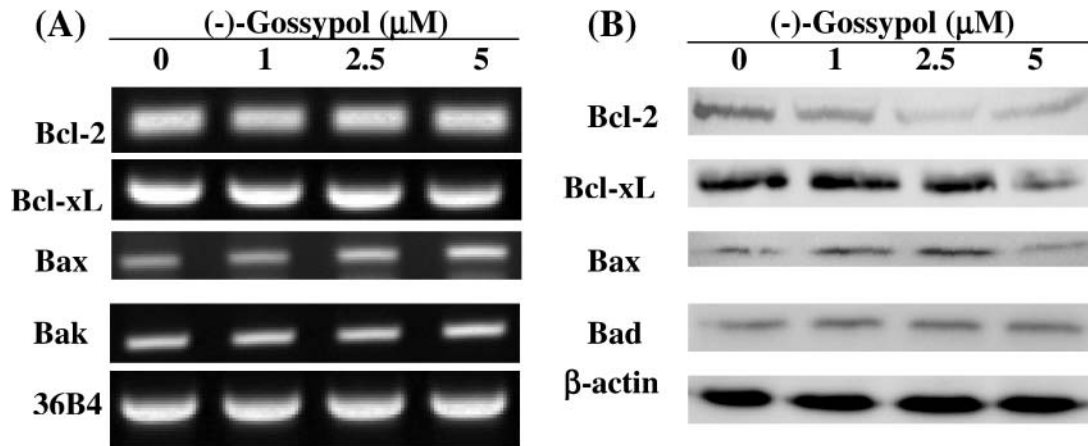
Figure 2. *Alterations of mRNA and protein expressions of the Bcl-2 family members in (-)-gossypol-treated DU-145 cells. DU-145 cells were treated with different concentrations of (-)-gossypol or 0.1% ethanol (vehicle control) for 24 h. (A) Potency expression of (-)-gossypol on mRNA levels of Bcl-2, Bcl-xL, Bax and Bak. 36B4 was used as an internal control. The mRNA expression was measured by densitometry of ethidium bromide-stained PCR products separated on a 1.5% agarose gel. (B) Fifty-microgram cell lysates were subjected to 4-15% gradient Tris-glycine gel electrophoresis, followed by immunoblot with antibodies against Bcl-2, Bcl-xL, Bax, and Bad, respectively. β-Actin probing was used to confirm equal loading of the total protein.*

Caspase-3 is believed to be the major caspase that functions as a downstream marker of caspase-9 and apoptosis. (-)-Gossypol reduced the full-form caspase-3 and activate the cleaved caspase-3 in a dose-dependent manner (Figure 3). PARP, an apoptotic molecule, is a DNA repair enzyme that can be cleaved by effector caspases. PARP was cleaved after treatment with (-)-gossypol from 1 to 5 μM. In comparison with the control, the levels of active PARP (89 kDa) increased by 40-65% (Figure 3). These results demonstrated that (-)-gossypol can also activate death substrates, such as PARP.

To investigate the mechanism behind (-)-gossypol-induced DNA fragmentation, the effects of (-)-gossypol on the DNA fragmentation factor (DFF) protein levels were examined in DU-145 cells. DFF is composed of two subunits, a 45-kDa subunit called ICAD (also designated as DFF45) and CAD (also designated as CPAN (caspase-activated nuclease) or DFF40). CAD is a DNase responsible for DNA degradation during apoptosis (22). ICAD functions as a regulatory subunit that inhibits CAD activity. Our results showed that (-)-gossypol treatment increased CAD protein expression in a dose-dependent manner; however, (-)-gossypol decreased the expression of full length ICAD protein (Figure 3). The cleavage of ICAD was very weak, suggesting that (-)-gossypol may induce ICAD cleavage and further degradation. These data indicated that CAD augmentation may be associated with (-)-gossypol-induced DNA fragmentation.

Caspase-dependent apoptosis of (-)-gossypol-treated DU-145 cells. Following the observation of apoptosis and changes in caspase protein expression induced by (-)-gossypol, our
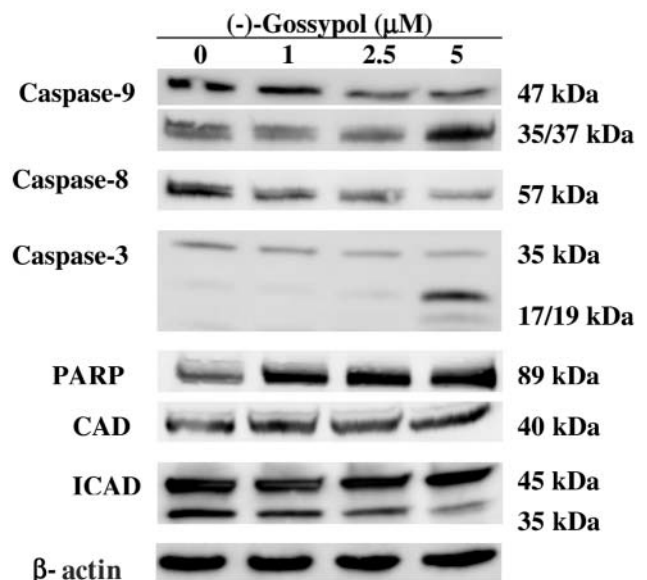

Figure 3. *Extensive caspase, PARP and CAD activation in (-)-gossypol-treated DU-145 cells. DU-145 cells were treated with different concentrations of (-)-gossypol or the vehicle as control for 24 h. Fifty-microgram cell lysates were subjected to 4-15% gradient Tris-HCl gel electrophoresis followed by immunoblot with antibodies of caspases-3, -8 and -9, PARP, CAD and ICAD, respectively. β-Actin reprobing confirmed equal loading of the total protein.*

attention turned to whether caspase activation was essential for (-)-gossypol-evoked apoptosis and which molecular pathways of apoptosis were modulated by (-)-gossypol.
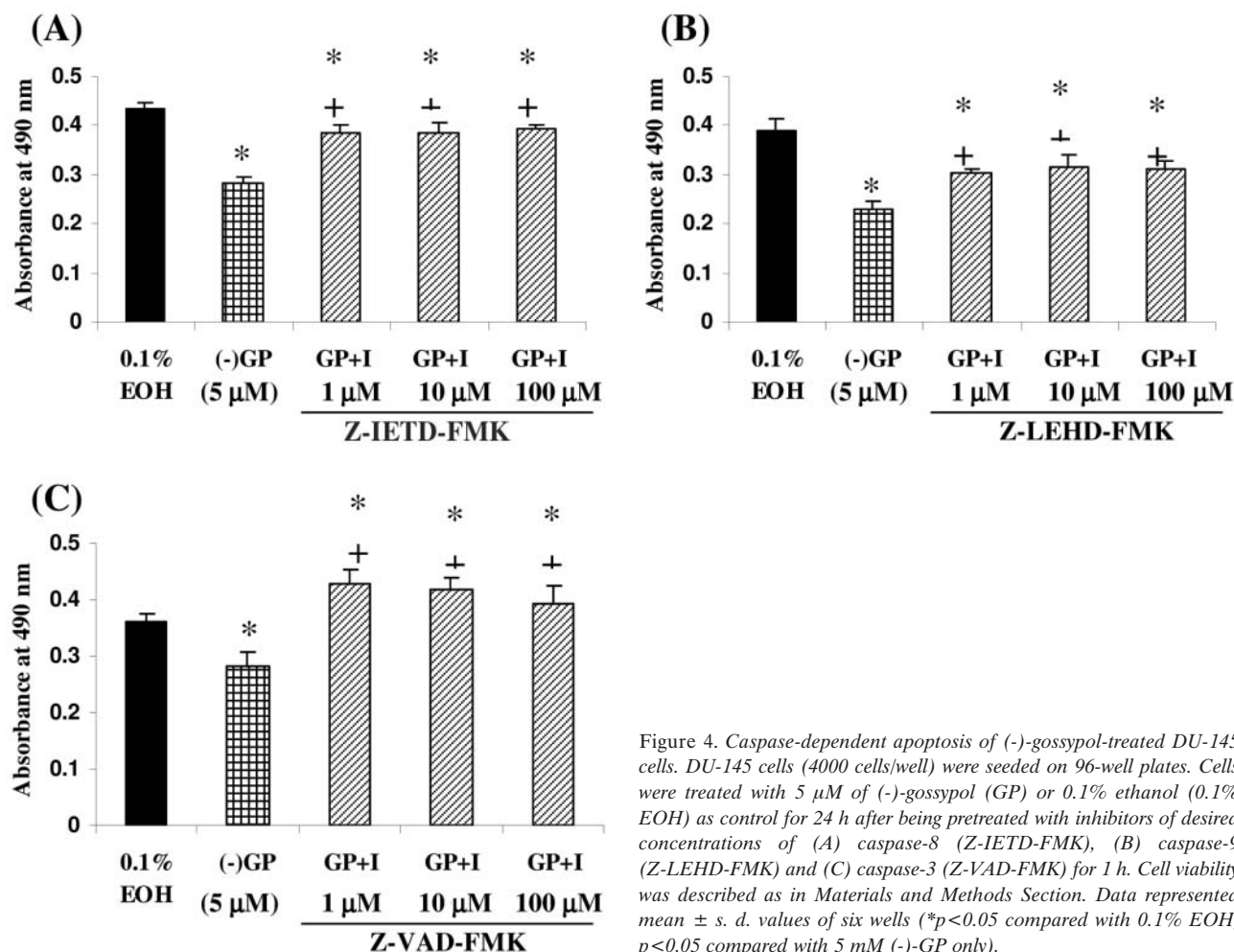
Figure 4. *Caspase-dependent apoptosis of (-)-gossypol-treated DU-145 cells. DU-145 cells (4000 cells/well) were seeded on 96-well plates. Cells were treated with 5 μM of (-)-gossypol (GP) or 0.1% ethanol (0.1% EOH) as control for 24 h after being pretreated with inhibitors of desired concentrations of (A) caspase-8 (Z-IETD-FMK), (B) caspase-9 (Z-LEHD-FMK) and (C) caspase-3 (Z-VAD-FMK) for 1 h. Cell viability was described as in Materials and Methods Section. Data represented mean ± s. d. values of six wells (*p<0.05 compared with 0.1% EOH; p<0.05 compared with 5 mM (-)-GP only).*

Different caspase inhibitors were used to block (-)-gossypol-inhibitory cell proliferation. The DU-145 cells were pretreated with various doses of inhibitors, including inhibitors of caspases-3, -8 and -9, for 1 h and were then exposed to 5 μM of (-)-gossypol for another 24 h. The cell viability assay by the MTS/PMS method indicated cell viability.

The relationship between caspase-8-mediated apoptosis *via* the death receptor pathway and (-)-gossypol-suppressed cell growth was examined first. When the cells were pretreated with Z-IETD-FMK, a cell-permeable inhibitor of caspase-8, from 1 to 100 μM, the cell viabilities significantly increased compared to cells treated with (-)-gossypol alone. However, the caspase-8 inhibitor did not completely block the inhibitory effects of (-)-gossypol when compared with vehicle-treated cells (Figure 4A). These results indicated that (-)-gossypol-induced apoptosis may result partially from caspase-8-mediated apoptosis and the death receptor pathway may also be involved.

The caspase-9 inhibitor, Z-LEHD-FMK, inhibits caspases -4, -5 and -9 (23) and is a mediator of the mitochondrial apoptosis pathway. The effects of treatment with Z-LEHD-FMK on DU-145 cells exposed to (-)-gossypol were tested as above. The results were similar to those obtained for the caspase-8 inhibitor. Moreover, the blocking efficacy of the caspase-9 inhibitor on (-)-gossypol-triggered cell apoptosis was more than 75%, indicating that the mitochondria-mediated apoptosis pathway was involved in (-)-gossypol-induced apoptosis (Figure 4B).

The caspase-3 inhibitor, Z-VAD-FMK, a general caspase inhibitor, blocked (-)-gossypol-induced cell death (Figure 4C). In contrast, cells pretreated with this inhibitor exhibited significantly reduced inhibition of cell growth due to (-)-gossypol. Increased cell growth was observed compared to cells treated with the vehicle (Figure 4C). All concentrations of caspase-3 inhibitor from 0.01 to 100 μM neutralized the effects of (-)-gossypol. Taken together, these results demonstrated that a caspase-dependent signaling pathway is involved in (-)-gossypol-induced apoptosis.

Discussion

The gossypols have been found to be active against a wide variety of cancer types, and have been shown to suppress multidrug-resistant cells and metastatic tumors (24, 25). Even the milk collected from gossypol-treated dairy cows has antitumor activity (26). In several clinical trials, the gossypols were shown to meet toxicity criteria and be well tolerated in patients with metastatic adrenal cancer (27), malignant gliomas (28, 29) and refractory metastatic breast cancer (24). Gossypol was reported to arrest cell cycle at the G_0/G_1-phase (18), this cell cycle redistribution being dictated either by cell cycle regulators or by TGF-β (4). Gossypol has also been demonstrated to inhibit the proliferation of cancer cells by inhibiting nuclear enzymes, such as DNA polymerase α and topoisomerase II (30), suppressing DNA synthesis (3), reducing protein kinase *C* activity (31), modulating the cell cycle regulatory proteins Rb and cyclin-D1 (24, 32), diminishing cellular energy metabolism (33), being directly toxic to mitochondria (34) or by blocking angiogenesis (35).

Recently, gossypol was reported to be a potent small molecule inhibitor of both Bcl-2 and Bcl-xL (15). The binding affinity of (-)-gossypol and (+)-gossypol to Bcl-2 is comparable (15), although (-)-gossypol is a more potent inhibitor of cell growth than either (+)-gossypol or racemic (±)-gossypol (12). Our study demonstrated that (-)-gossypol can alter the Bcl-2 family expression, especially in down-regulating both the mRNA and protein expressions of Bcl-2 and Bcl-xL and up-regulating Bax and Bak expressions. The Bcl-2 family members all contain a BH3 domain (13); therefore, it has been proposed that (-)-gossypol, acting as a non-selective BH3 mimetic, may bind to the BH3 binding pocket of various Bcl-2 family members (16). This binding would activate Bak or Bax directly and promote cell death, also simultaneously inhibiting anti-apoptotic members (Bcl-2 or Bcl-xL) (16). More recently, (-)-gossypol was reported to act directly on the mitochondria to overcome Bcl-2- and Bcl-xL-mediated apoptosis resistance in Bcl-2/Bcl-xL-transfected Jurkat T-cells; this effect is associated with Bak activation and the release of cytochrome *c* (16). Bak activation might correlate with the expression of anti-apoptotic members, since pro-apoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not by Bcl-2, until Bak is displaced by BH3-only proteins (36). Gossypol induced apoptosis in human leukemia cells (HL-60) and caused truncation of the Bid protein, the loss of mitochondrial membrane potential, cytochrome *c* release from the mitochondria into the cytosol, and activation of caspases-3, -8 and -9 through the ROS (reactive oxygen species)-independent mitochondrial dysfunction pathway (37). Gossypol was also found to up-regulate Fas and FasL associated with downstream caspase-8

activity in lung cancer cells (19). These findings suggest that gossypol can induce apoptosis by at least two pathways: one mechanism targets the Bcl-2 family, particularly Bcl-2 and Bcl-xL, associated with the mitochondrial pathway and the other mechanism results in the activation of death receptor-associated signal transduction.

Our results indicated that (-)-gossypol induced caspases-3 and -9 activities. Although the active form of caspase-8 was not observed after (-)-gossypol treatment, a decrease of full-length caspase-8 was detected. Caspase-8 activity is associated with extrinsic apoptotic pathways triggered by engagement of the cell surface death receptor. We speculate that (-)-gossypol down-regulated uncleaved caspase-8 expression leading to protein degradation. Caspase-9 activity is associated with the intrinsic pathway of apoptosis and activated caspase-9 acts on downstream caspase-3. Our data suggested that activated caspase-3 may further cleave PARP and ICAD, resulting in increased CAD expression. Furthermore, our findings indicated that (-)-gossypol-triggered DNA fragmentation might be partially caused by increased CAD expression.

Gossypol was found to activate caspases-3, -6, -7, -8 and -9 (18, 37) and caspase inhibitors were used to investigate whether (-)-gossypol-induced apoptosis was caspase-dependent and/or -independent. We demonstrated that caspases-8 and -9 inhibitors only partially blocked the (-)-gossypol-associated reduction in cell viability. However, a caspase-3 inhibitor totally blocked the inhibition of cell growth by (-)-gossypol. Our results are consistent with those of others (18, 37) who indicated gossypol-induced apoptosis, not only through the mitochondrial pathway, but also *via* the death receptor pathway, which is caspase-dependent.

One of the important benefits of using gossypol as an anticancer drug is its ability to suppress multidrug-resistant cancer cells (2, 3, 25). Although some reports have stated that serum may affect the anticancer activity of gossypol *in vitro* and *in vivo* (38-40), we found that gossypol had a greater ability to bind α-lactoalbumin than other milk proteins (unpublished data) and that milk collected from gossypol-treated dairy cows retained anticancer activity (26). Furthermore, gossypol is transferred *via* milk from the nursing dams to their neonates (41). In conclusion, (-)-gossypol may have chemopreventive benefits by enhancing cancer cell apoptosis in prostate cancer patients. Therefore, the consumption of (-)-gossypol-containing milk or snacks made from cottonseed oil or cottonseed meal flour may be of therapeutic benefit to human cancer patients. In addition, we further speculate that the development of (-)-gossypol-enriched cottonseed oil for daily human consumption may also exert chemopreventive effects in healthy individuals. Studies examining the mechanisms of (-)-gossypol down-regulated Bcl-xL in prostate cancer cells are ongoing in our laboratory.

Acknowledgements

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