Effects of Serum on (–)-Gossypol-suppressed Growth in Human Prostate Cancer Cells

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Abstract. *Background: Gossypol, a natural polyphenolic compound present in cottonseeds, possesses antiproliferative and pro-apoptotic effects in in vivo and in vitro models. There are two enantiomers, (+)-gossypol and (–)-gossypol, the latter being a more potent inhibitor of cancer cell growth. Here, the effect of bovine serum albumin (BSA) and dextran-coated charcoal-treated fetal bovine serum (DCC-FBS)-containing medium on the ability of (–)-gossypol to inhibit the growth of human prostate cancer cells was studied. Materials and Methods: BSA- and DCC-FBS-supplemented medium were used to examine the influence of serum proteins on the antiproliferative effects of (–)-gossypol in DU-145 cells, a human prostate cancer cell line. The viability of the DU-145 cells was determined by CellTiter 96™ Aqueous assay. The expressions of mRNA and protein for the cell cycle regulators, cyclin-D1, Rb, CDK, p21 and TGF-β, were determined by RT-PCR and Western blot analyses, respectively. Results: (–)-Gossypol caused growth suppression of the DU-145 cells. In comparison with BSA-supplemented medium, DCC-FBS blocked the antiproliferative effects of (–)-gossypol at 1 and 2.5 μM, but not at 5 μM. Furthermore, (–)-gossypol treatment down-regulated cyclin-D1, Rb, CDK4 and CDK6, and up-regulated p21 and TGF-β1 at the mRNA and/or protein levels. Conclusion: The data suggested that (–)-gossypol-suppressed prostate cancer cell growth may be influenced through cell cycle regulators, which may lead to better prognosis. We further speculate that (–)-gossypol might serve as a chemotherapeutic agent for human prostate cancer patients.*

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In 2006, the American Cancer Society estimates that prostate cancer will account for one of every three cancer diagnoses in American men (1), and is estimated as the third cause of cancer death after lung and colon cancer in man (1).

Gossypol (*Gossypium L., Malvaceae*), a naturally occurring polyphenolic pigment, is present in cottonseeds and by-products, such as cottonseed oil and cottonseed meal flour, frequently consumed by humans and food-producing animals. Previously, research on gossypol in relation to cancer had focused mainly on the antiproliferative activity of racemic gossypol, (±)-gossypol, in a variety of cancers *in vitro* and *in vivo* including breast, ovary, cervix, uterus, adrenals, pancreas, colon, head and neck. Studies from our laboratory demonstrated the antiproliferative effects of (±)-gossypol on human breast cancer cells (2, 3), and anticancer activity in human prostate (4, 5), canine prostate (6, 7) and rat prostate cancer (8). These anticancer effects have been attributed to the (–)-enantiomer, which is a more potent inhibitor of cancer cell growth (9, 10). Our previous results demonstrated that (–)-gossypol is more effective in inhibiting the growth of human adipose stromal cells (11) and human breast cancer cells (12).

The anticancer properties of gossypol have been associated with modulation of TGF-β (4), while its antimetastatic ability has been attributed to modulation of the expression of the metastatic suppressor gene, nm23 (8). Treatment of cells with gossypol inhibited DNA synthesis (4), caused DNA breaks (13), induced DNA fragmentation, and repressed protein kinase *C* activity (14). The antitumor and antimitotic activities might also result from inhibition of human mitotic kinesin Eg5 (15), vertebrate inositol-1,4,5-trisphosphate 3-kinases, inositol polyphosphate multikinase (16) and telomerase (17). Zhang *et al.* found that gossypol suppressed cell proliferation through the induction of p21 (18). Moreover, our findings, as well as those of others, indicated that gossypol modulates cell growth *via* the regulation of retinoblastoma (Rb) and cyclin-D1 expression in cancer cells (4, 19).

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

Gene	Primer sequences	Anneal (°C)	MgCl$_2$ (mM)	Cycle number	Product size (bp)
Cyclin-D1	5'-GCTCCTGTGCTGCGAAGTGG-3'				
	5'-TGGAGCCGTCGGTGTAGATG-3'	50	2.5	28	383
p21	5'-AGGCGCCATGTCAGAACCGGCTGG-3'				
	5'-GGAAGGTAGAGCTTGGGCAGGC-3'	63	1.0	30	220
Rb1	5'-CTCTCGTCAGGCTTGAGTTT-3'				
	5'-ATGGACACTGATTTCTATGT-3'	58	1.5	30	260
TGF-β1	5'-CAAGACCATCGACATGGAGCTGGTGA-3'				
	5'-CAGTTCTCCGTGGAGCTGAAGCA-3'	60	2.0	30	563
36B4	5'-AAACTGCTGCCTCATATCCG-3'				
	5'-TTGATGATAGAATGGGGTACTGAT G-3'	63	3.5	30	563

In the present study, the molecular mechanisms of (–)-gossypol involved in cancer cell growth inhibition, and the effect of bovine serum albumin (BSA) and dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) on the anticancer properties of (–)-gossypol were investigated in prostate cancer cells. Our results demonstrated that (–)-gossypol reduced cell growth, but, importantly that serum proteins of DCC-FBS reduced the anticancer effect of (–)-gossypol. Prostate cancer cell growth inhibition by (–)-gossypol was associated with down-regulation of cyclin-D1, Rb, CDK4 and CDK6 at both the mRNA and/or protein levels. Our observations suggested that consumption of (–)-gossypol-containing cottonseed products 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), and the cells were maintained in a mixture of DMEM and F12 without phenol red (Atlanta Biologicals, Norcross, GA, USA) supplemented with 5% FBS and antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (Invitrogen, Carlsbad, CA, USA).

Antibodies against cyclin-D1, Rb, p21, CDK4 and CDK6 were purchased from Cell Signaling Technology (Beverly, MA, USA), white antibodies against β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase (HRP)-linked antibodies were purchased from GE Healthcare (Piscataway, NJ, USA). (–)-Gossypol was provided by the co-author Dr. Michael Dowd from the USDA, Southern Regional Research Center (New Orleans, LA, USA); its preparation has been described in detail previously (20).

Cell viability assay. The effects of (–)-gossypol and the serum proteins of BSA and DCC-FBS on cell viability were assessed by the CellTiter 96™ 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 sextuple wells in phenol red-free DMEM/F12 supplemented with 5% FBS overnight. The medium then was switched to DMEM/F12 supplemented with either DCC (Dextran T-70, Pharmacia; activated charcoal, Sigma)-stripped FBS (5%) or 0.2% BSA, to investigate their influence on the anticancer effect of (–)-gossypol.

To measure the effect of (–)-gossypol on cell viability, DU-145 cells were treated with (–)-gossypol at 1 to 5 μM or 0.1% ethanol as the vehicle control in fresh medium (BSA or DCC group) for 24 h. At the end of treatment, cell viability was measured by adding a mix of 20 μl freshly combined MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and PMS (phenazine methosulfate) (ratio 20:1) solution to each well. The plates were 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. The results were expressed as the ratio of viable-treated cells to vehicle treated cells in a multi-well plate format.

RNA isolation and RT-PCR analysis. A total of 1x10^5 cells was seeded in each well of a six-well plate with 2 ml of culture medium in the presence or absence of (–)-gossypol (1-5 μM). Total RNA was isolated using 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 dithiothreitol (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. 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 amplification conditions of the cell cycle-related genes (cyclin-D1, p21, Rb1 and TGF-β1) 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
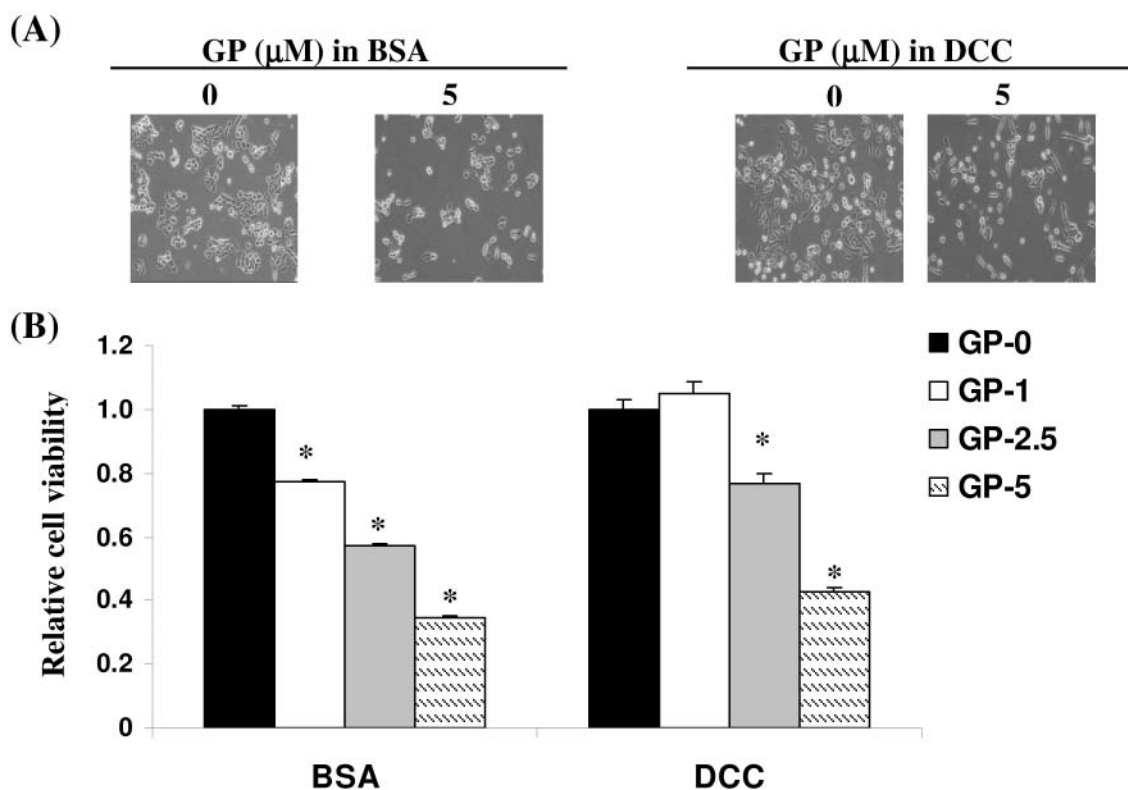
Figure 1. *Effect of serum proteins on (–)-gossypol-suppressed viability of human prostate cancer cells, DU-145. (A). The morphology of DU-145 cells treated with 0.1% ethanol (GP-0) vehicle or 5 μM (–)-gossypol in medium containing either 0.2% BSA (BSA group) or 5% DCC-FBS (DCC group) for 24 h. Light microscopic examination indicated that (–)-gossypol-treated cells underwent pronounced morphological changes. The cells became shrunken, round and detached from the flask. (B). Cancer cells (4,000 cells/well) were grown in 96-well plates with 0.2% BSA or 5% DCC-FBS for 24 h, then were exposed to the indicated concentrations of (–)-gossypol or 0.1% ethanol (vehicle control, GP-0) for 24 h. The cell density was assessed colorimetrically after addition of MTS/PMS and expressed as an optical density ratio of the treatment to control at 490 nm. Each bar represents the mean ± s.d. of six wells. The asterisks show significant differences from the control group (p<0.05).*

bromide. The specific bands of mRNA expression were measured by densitometry of the 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 phosphate-buffered saline (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 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 10% SDS-polyacrylamide 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 PBS containing 0.1% Tween-20 (PBST) and then blocking with PBST containing 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, the primary antibodies being diluted 1:1,000.

Following incubation in the primary antibody, the membranes were rinsed briefly and washed twice with PBST for a total of 20 min, and then incubated in goat anti-rabbit or anti-mouse IgG-HRP conjugates (diluted 1:5,000) for 1 h at room temperature. After the second incubation, the membranes were rinsed and washed as before. Immunoblots were enhanced by ECL Plus reagent (GE Healthcare, USA) 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 the mRNA expression of the cell cycle regulators were presented as one group by the mean ± standard deviation (s.d.) for three or six replicate culture wells, respectively. Analysis was performed using the StatView® ANOVA unpaired *t*-test. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Effect of serum on (–)-gossypol-reduced cell viability in human prostate cancer cells. To address the anticancer effect of (–)-gossypol on prostate cancer cell growth, DU-145 cells
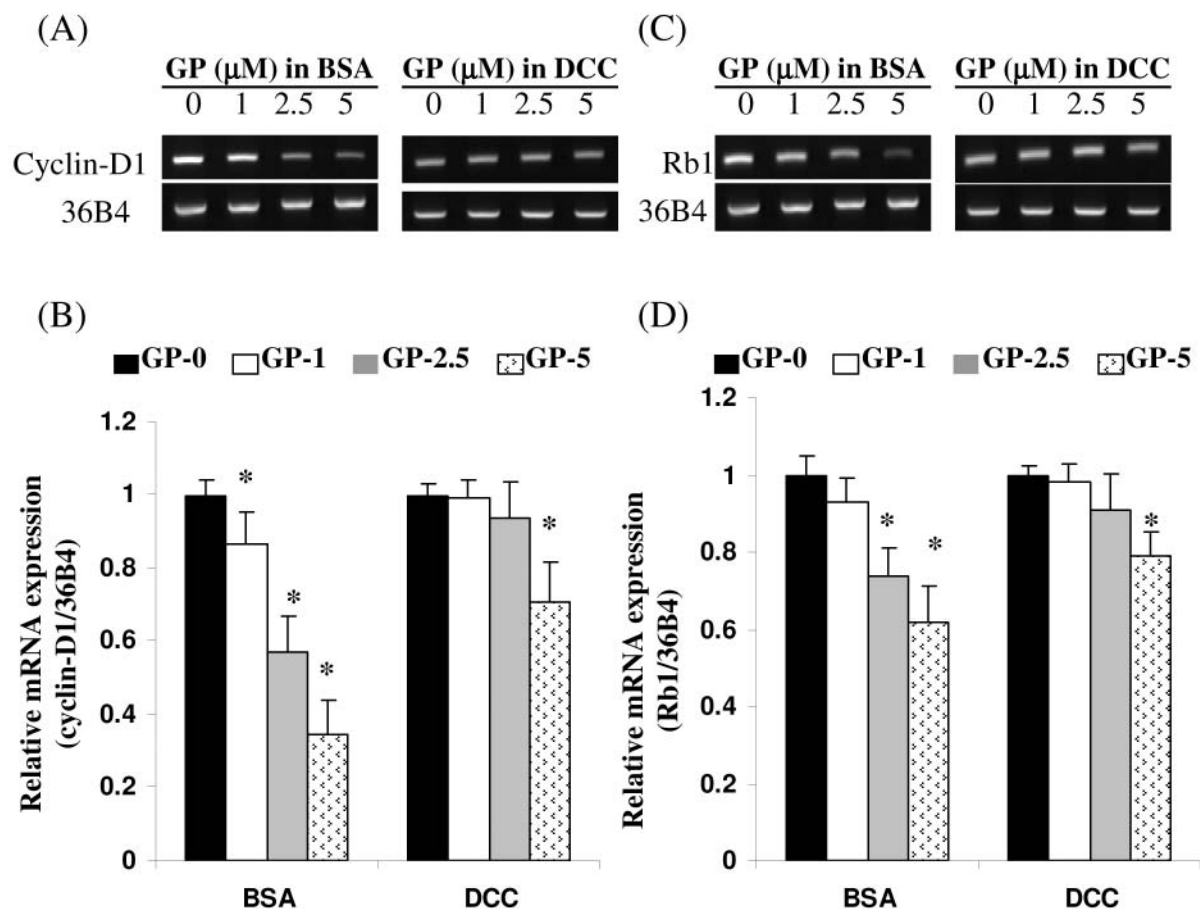
Figure 2. (–)-Gossypol down-regulated cyclin-D1 and Rb1 mRNA expression in human prostate cancer cell line, DU-145 cells. The cancer cells were grown in medium containing 0.2% BSA or 5% DCC-FBS (DCC) and then exposed to the indicated concentrations of (–)-gossypol (GP) or 0.1% ethanol (GP-0) as control for 24 h. (A) and (C) represent cyclin-D1, Rb1 and 36B4 mRNA expressions of ethidium bromide-stained PCR products separated on a 1.5% agarose gel. Total RNA was isolated from each treatment and RT-PCR was performed as described in the Materials and Methods section. 36B4 was used as an internal control. (B) and (D) represent the relative cyclin-D1 and Rb1 mRNA ratios measured by densitometry. The bars represent means ± s.d. (n=3). The asterisks show significant differences from the control (GP-0) (p<0.05).

were treated with 1 to 5 μM (–)-gossypol or vehicle for 24 h. The results showed that (–)-gossypol treatment altered the morphology of the DU-145 cells cultured in either BSA- or DCC-FBS-containing medium; the cells became shrunken, round and detached from the flasks (Figure 1A). The viability of the DU-145 cells was significantly reduced by (–)-gossypol treatment in a dose-dependent manner in both BSA- and DCC-FBS-containing medium (Figure 1B). However, it was observed that (–)-gossypol was more effective in reducing DU-145 cell viability in BSA-supplemented medium (Figure 1B). The DCC-FBS resulted in removal of most of the steroid hormones, but not the growth factors which may counter the antiproliferative effects of (–)-gossypol. When cultured in DCC-FBS-supplemented medium, treatment with (–)-gossypol at 2.5 and 5 μM significantly reduced DU-145 cell viability by 25%

and 58%, respectively (Figure 1B). These data indicated that growth factors and serum proteins other than BSA affected the anticancer effects of (–)-gossypol.

(–)-Gossypol down-regulated cyclin-D1, Rb, CDK4 and CDK6, but up-regulated TGF-β1 and p21 expressions in human prostate cancer cells. Our previous results and those of others determined that (±)-gossypol induced cell cycle arrest at the G_0/G_1-phase (18, 21). To investigate the antiproliferative activity of (–)-gossypol on the expression of cell cycle regulatory proteins, DU-145 cells were treated with (–)-gossypol in either BSA- or DCC-FBS-containing medium for 24 h. mRNA expression was measured by RT-PCR, and protein expression determined by Western blot analysis. It was observed that (–)-gossypol in BSA-supplemented medium more effectively down-regulated
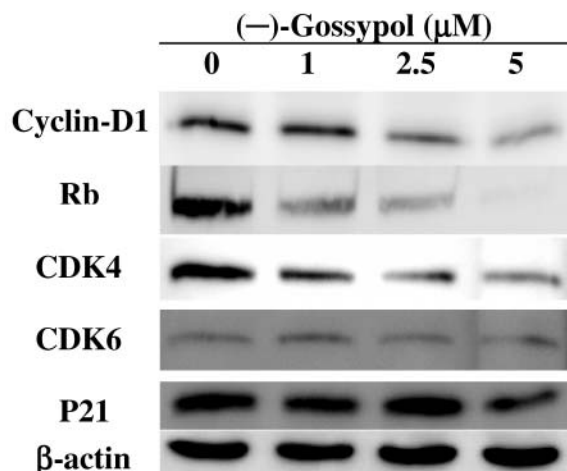
Figure 3. *Alternation caused by (−)-gossypol on cell cycle regulators in human prostate cancer cell line, DU-145. Cancer cells were treated with 1, 2.5 and 5 μM of (−)-gossypol or vehicle control in medium containing 5% DCC-FBS for 24 h. Then, the cells were collected and the total proteins isolated for Western blot analysis. The total proteins (50 μg) were subject to a 10% Tris-HCl gel electrophoresis followed by immunoblot with antibodies against cyclin-D1, Rb, CDK4, CDK6 and p21, respectively. β-actin reprobing was used to confirm equal loading of the total protein.*

cyclin-D1 and Rb1 mRNA expressions, although in DCC-FBS-containing medium, 5 μM (−)-gossypol treatment significantly decreased both cyclin-D1 mRNA and protein levels (Figures 2 and 3). Similar findings were observed for Rb1 mRNA and Rb protein expression, which was diminished by (−)-gossypol treatment in a dose-dependent manner (Figures 2 and 3). At 5 μM (−)-gossypol treatment, Rb protein was almost undetectable (Figure 3). These observations indicated that (−)-gossypol inhibited cancer cell proliferation *via* suppression of cyclin-D1 and Rb expression, which is consistent with our own and others previous findings (8, 18, 19).

After observing the reduction of cyclin-D1 expression by (−)-gossypol, the changes in CDK (cyclin-dependent kinase) expression were examined. Cyclin-D1, an important regulatory protein at the checkpoint from G_1- to S-phases of the cell cycle, binds with CDK4 and CDK6. Our results illustrated that the levels of CDK4 and CDK6 proteins were decreased by (−)-gossypol treatment (Figure 3). Therefore, inhibition of the expression of G_1 check-point regulators, CDK4 and CDK6, in (−)-gossypol-treated DU-145 cells might result in cell cycle arrest. To explore this mechanism further, the expression of p21, a CDK inhibitor, in response to (−)-gossypol treatment was examined. The data demonstrated that 5 μM (−)-gossypol in both BSA- and DCC-FBS-containing medium significantly increased p21 mRNA expression (Figure 4). p21 protein expression was up-regulated by (−)-gossypol at 2.5 μM treatment, but down-regulated by (−)-gossypol at 5 μM (Figure 3). Functionally, p21 is thought to stabilize the interaction between CDK4/CDK6 and D-type cyclins, thereby, promoting the formation of active complexes in a concentration-dependent manner (22). Our results demonstrated that (−)-gossypol treatment increased p21 expression, which was correlated with cell cycle arrest, because induction of p21 predominantly leads to cell cycle arrest (23).

Our previous findings indicated that the inhibitory effects of (±)-gossypol on cancer cell proliferation are associated with the induction of TGF-β1 (4, 5). In the present study, we also investigated TGF-β1 mRNA expression after (−)-gossypol treatment in DU-145 cells. Interestingly, it was observed that TGF-β1 mRNA was augmented more by (−)-gossypol at 5 μM in DCC-FBS-supplemented medium compared to BSA-supplemented medium (Figure 4). These results indicate that the antiproliferative effects of (−)-gossypol on DU-145 cells are mediated through disruption of the cell cycle by decreasing cylin-D1, Rb, CDK4 and CDK6, but by increasing p21 and TGF-β1 expressions. The possible mechanisms of (−)-gossypol interference with the cell cycle might be linked to p21 and TGF-β induction, in turn reducing cyclin-D1 and CDK4/CDK6. Cyclin-D1 can form a complex with either CDK4 or CDK6 and is involved in the phosphorylation of the Rb gene product which is pivotal in controlling progress through the cell cycle (19).

Discussion

Gossypol has been found to be active against a wide variety of cancers, and has been shown to suppress multidrug-resistant cells and metastatic tumors (19, 24). In several clinical trials including patients with metastatic adrenal cancer (25), malignant gliomas (26, 27) and refractory metastatic breast cancer (19), gossypol met the toxicity criteria and was well tolerated. Gossypol was reported to arrest cell cycle at the G_0/G_1-phase (18, 21), this cell cycle redistribution being dictated by cell cycle regulators or by TGF-β (4). Gossypol also has been demonstrated to suppress the proliferation of cancer cells *via* inhibition of nuclear enzymes, such as DNA polymerase α and topoisomerase II (28), suppression of DNA synthesis (3), reduction of protein kinase C activity (29), modulation of the cell cycle regulatory proteins Rb and cyclin-D1 (19, 30), diminution of cellular energy metabolism (31), direct toxicity to mitochondria (32), or blocking of angiogenesis (33).

Our current studies demonstrated that (−)-gossypol reduced the viability of DU-145 prostate cancer cells *in vitro*. The molecular mechanisms appeared to involve the down-regulation of Rb and cyclin-D1 at both the mRNA and protein levels. CDK4 and CDK6 protein expressions
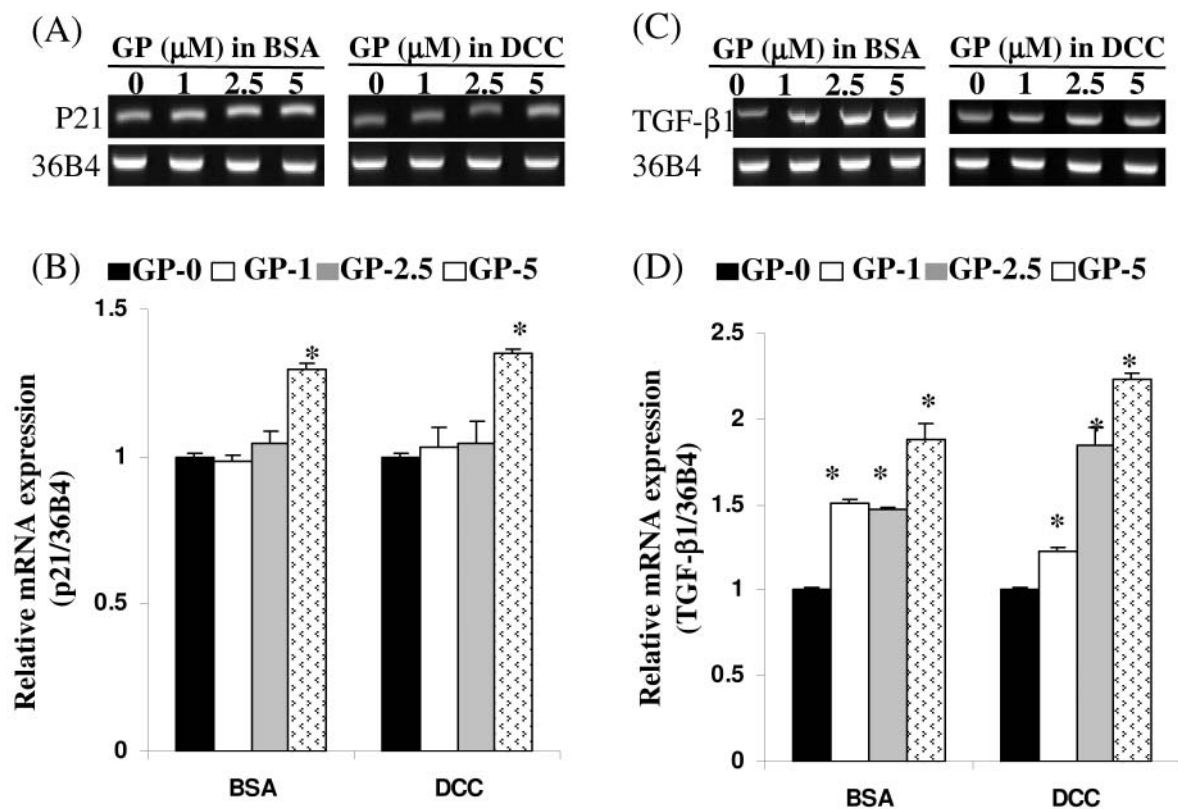
Figure 4. (–)-Gossypol up-regulated p21 and TGF-β1 mRNA expressions in human prostate cancer cell line, DU-145 cells. Cancer cells were grown in medium containing 0.2% BSA or 5% DCC-FBS (DCC) and then exposed to the indicated concentrations of (–)-gossypol (GP) or 0.1% ethanol (GP-0) as control for 24 h. (A) and (C) represent p21, TGF-β1 and 36B4 mRNA expressions of ethidium bromide-stained PCR products separated on a 1.5% agarose gel. The total RNA was isolated from each treatment and RT-PCR was performed as described in the Materials and Methods section. 36B4 was used as an internal control. (B) and (D) represent the relative p21 and TGF-β1 mRNA ratios measured by densitometry. Bars as means ± s.d. (n=3). The asterisks show significant differences from the control (GP-0) (p<0.05).

were also reduced by (–)-gossypol treatment. Further, p21 mRNA was up-regulated by low doses of (–)-gossypol, while TGF-β1 mRNA was increased.

It has been proposed that gossypol-induced cell cycle arrest in human breast cancer cells is connected to the regulation of cyclin-D1 and Rb (19). Our results in human prostate cancer cells agree with this proposed mechanism, although DU-145 cells do not express wild-type p53. In colon cells, it was found that p53 and Rb were not altered by gossypol treatment and p21 might be the very first target of gossypol to initiate cell growth inhibition of HT-29 cells (18). p21 is a transcriptional target of p53 that plays a crucial role in mediating growth arrest displaying p53-dependent or -independent repression (23). Our findings indicated that (–)-gossypol up-regulated p21 expression might occur through a p53-independent pathway, because DU-145 cells do not express wild-type p53. Conversely, (–)-gossypol-mediated regulation of p21 might occur through regulation of TGF-β1 expression, because there is a

response element within the p21 promoter region regulated by TGF-β (23). Up-regulation of p21 by (–)-gossypol might further inhibit CDK4/CDK6 and cyclin-D1 expressions, although, cannot be excluded the possibility that (–)-gossypol directly inhibits cyclin-D1 and CDK4/CDK6 expressions. Based on these results and observations, (–)-gossypol probably affects multiple targets on cell cycle-regulatory proteins, which culminate in repression of cell growth in human prostate cancer cells.

It is worth noting that the antiproliferative effect of (–)-gossypol at 1 μM was blocked by DCC-FBS (Figure 1B). Similar effects of serum proteins against the action of gossypol (3) have been reported with BCL-D1 human diploid fibroblasts, murine erythroleukemia cells and bovine luteal cells. Our previous study showed that suppression of DNA synthesis by racemic (±)-gossypol was blocked by BSA in human breast cancer cells (3). Additional evidence indicated that (±)-gossypol, especially the (+)-gossypol enantiomer, could bind to serum proteins, which would

reduce the effective dose of (±)-gossypol and the (–)-gossypol enantiomer (3). Accordingly, (–)-gossypol has been suggested to be a more potent anticancer agent than (±)-gossypol or (+)-gossypol (3). It was previously shown that (±)-gossypol selectively damaged the periportal region of lobules in perfused rat liver; however, the addition of BSA to the perfusate completely prevented the toxic effect of (±)-gossypol on hepatocytes and decreased the absorption of (±)-gossypol by the liver (34). Thus, this earlier study indicated that rat liver could be protected by (±)-gossypols binding to BSA (34). Our current results demonstrated that (–)-gossypol may also bind to DCC-FBS serum proteins other than BSA, resulting in the observed decreased biological activity of (–)-gossypol (Figures 1-4). Although DCC-FBS blocked the anticancer activity of (–)-gossypol at lower doses (1 and 2.5 μM treatment, Figures 1-4), (–)-gossypol at 5 μM treatment significantly counteracted the blocking effect of serum proteins. This result was consistent with our previous finding demonstrating that milk collected from gossypol-treated mid-lactation Brown Swiss dairy cows (35) still exerted antitumor activity (36) and that the antiproliferative potential of gossypol-containing milk might serve as a dietary supplement for the prevention and/or treatment of human cancer. Moreover, milk protein-bound gossypol passed *via* milk from nursing dams to neonates and free gossypol could be extracted and detected in the stomachs of the neonates, as well as in the blood and other organs (37). These data demonstrated that protease enzymes in *in vivo* models could cleave the protein-bound gossypol to release free gossypol with an active anticancer function.

In conclusion, (–)-gossypol may have chemopreventive benefits by causing cell cycle arrest in prostate cancer cells. The consumption of (–)-gossypol-containing milk or snacks from cottonseed oil or cottonseed meal flour may be of therapeutic benefits in cancer patients. In addition, the development of (–)-gossypol-enriched cottonseed oil or cottonseed meal flour for human daily consumption might also serve as a chemopreventive agent in healthy individuals.

Acknowledgements

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