The Inhibitory Effects of Gossypol on Human Prostate Cancer Cells-PC3 are Associated with Transforming Growth Factor Beta$_1$ (TGFβ_1) Signal Transduction Pathway

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Abstract. *Background: Racemic gossypol [(±)-GP], a naturally occurring polyphenolic yellow pigment present in cottonseed products, inhibits in vitro proliferation of Dunning prostate cancer cells (MAT-LyLu), human prostate cancer cells derived from a bone marrow metastasis (PC3), MCF-7 and primary cultured human prostate cells. (±)-GP also has the ability to inhibit the metastasis of lung and lymph nodes of the androgen-independent rodent prostate cancer cell line, MAT-LyLu, after implantation into Copenhagen rats. Materials and Methods: The effects of (±)-GP on the proliferation of human prostate cancer PC3 cells were determined by thymidine incorporation assay and doubling-time (DT) determination. The mechanisms of action of (±)-GP on the proliferation of PC3 cells were determined by RT-PCR analysis, ELISA assay and Western blot analysis. Results: The results show that (±)-GP caused reductions in DNA synthesis and prolonged the DTs in PC3 cells. RT-PCR and ELISA results show that (±)-GP elevate the mRNA expression and protein secretion of transforming growth factor beta$_1$ (TGFβ_1) in PC3 cells. Consistent with these findings, (±)-GP has been shown to decrease the cyclin D$_1$ mRNA expression and protein expression in PC3 cells. Furthermore, the growth inhibition of PC3 cells by conditioned media collected from the (±)-GP-treated-PC3 cells was completely reversed by addition of 25μg/ml of mouse monoclonal anti-TGFβ_1,-β_2,-β_3 antibody, suggesting the involvement of TGFβ_1 in (±)-GP-induced growth inhibition of PC3 cells. Conclusion: These results indicate that the inhibitory effects of (±)-GP on the proliferation of human prostate cancer PC3 cells are associated with induction of TGFβ_1, which in turn influences the expression of the cell cycle-regulatory protein, cyclin D$_1$, in prostate cancer cells.*

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Prostate cancer is the most common malignancy in men and is the second leading cause of male cancer death in the U.S. (1, 2). Previous research has shown that androgens, such as testosterone and dihydrotestosterone, stimulate the growth of this malignancy and are involved in prostate cancer pathogenesis (3,4). The mechanism of prostatic carcinogenesis and tumorigenesis probably involves a multi-step progression from precancerous cells to cells which are proliferative and metastatic (5). The growth and development of prostate cancer cells appear to be androgen-dependent initially (6), with the androgens acting through their receptors (androgen receptors [AR]) to regulate the transcription of downstream genes controlling cellular growth and differentiation (7). Androgen deprivation and antiandrogens inhibit the AR's transcriptional function, thus suppressing its ability to act as a transcription factor. This results in the blockade of the survival signals elicited by androgens and the subsequent induction of apoptosis (8,9). Therefore, androgen deprivation is the primary treatment method for prostate cancer (7,10). Androgen withdrawal initially may reduce the growth of metastatic prostate cancers; however, the long-term endocrine treatment of prostate cancer patients always results in loss of responsiveness. Prostate cancer cells lose androgen dependency during the course of

cancer progression and become androgen-independent so that androgen deprivation therapy is unsuccessful (7, 8).

Racemic gossypol [(±)-GP] is a naturally occurring yellow pigment present in cottonseed products consumed by humans and food-producing animals (11). (±)-GP has been shown to be an extremely active compound that exerts a variety of effects in both *in vivo* and *in vitro* model systems relevant to the regulation of control mechanisms underlying normal and diseased conditions. (±)-GP has been demonstrated to be a potent antifertility agent in both males (12-14) and females (15-18). More recently, (±)-GP has generated research interest for its anticancer activity. In fact, the National Institutes of Health (NIH) has patented (±)-GP for treatment of human cancer patients (19). Research results have shown that (±)-GP inhibits the proliferation of many human cancer cells *in vitro* and *in vivo* (20-22). A significant body of evidence indicates GP's anti-cancer and anti-proliferative effects on a variety of human cancer cell lines, including those of the breast, prostate, ovary, cervix, uterus, adrenals, pancreas and colon (23-30). In Copenhagen rats that were recipients of transplanted MAT-LyLu prostate cancer tissue, (±) -GP at 12.5 mg/kg body weight per day for 14 days significantly reduced tumor weight and serum testosterone levels. It also significantly reduced the metastasis in both lymph nodes and lungs of (±)-GP-treated MAT-LyLu-bearing Copenhagen rats (20). Differential cytotoxicity of enantiomers of (±)-GP has been observed in a variety of human cancer cell lines. (-)-GP has been reported to be more cytotoxic than (±)-GP and (+)-GP in human skin fibroblasts (31), melanoma cell line (32), breast cancer cell lines (33, 34) and ovarian cancer cell lines (24).

Although the precise mechanism of action of (±)-GP is still unknown, (±)-GP has been shown to inhibit some enzymes involved in steriodogenesis such as 5α-reductase and 3α-hydroxysteroid dehydrogenase (35). Our results demonstrate that (±)-GP acts *via* a not-yet-defined mechanism to exert its anti-proliferative and anti-metastatic effects on prostate cancer cells. (±)-GP arrests the cell cycle of androgen-independent human prostate cancer cell line, PC3 and primary cultured cells isolated from benign prostatic hyperplasia (BPH) tissue and human breast tissue in association with increases in the expression of $TGF\beta_1$ mRNA (21, 36, 37), suggesting the involvement of $TGF\beta_1$ in (±)-GP -induced growth inhibition. $TGF\beta_1$ is a potent inhibitor of epithelial cell growth and has been shown to mediate the anti-proliferative effects of many anti-tumor agents such as vitamin D_3 (38) and tamoxifen (39). TGF β_1 exerts its effects by binding to a cell surface receptor and triggering a signaling pathway that regulates factors involved in the cell cycle such as Rb, cyclin and cyclin-dependent kinase (40). The reported experimental data are limited and little is known about the mechanisms of action of (±)-GP on prostate cancer cells. We investigate the anti-proliferative activities and potential mechanisms of (±)-GP in PC3 prostate cancer cell line.

Materials and Methods

Reagents. Racemic gossypol [(±)-GP] (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in dimethylsulphoxide (DMSO) to make a 10 mM stock solution. Treatment solutions were prepared by the dilution of stock solution in culture medium. Ultrapure natural human $TGF\beta_1$ and mouse monoclonal anti-TGF-β_1,-β_2,-β_3 antibody was purchased from Genzyme Corp. (Cambridge, MA, USA).

Cell culture. The PC3 human prostate cancer cell line was originally obtained from the American Type Tissue Culture Collection (Bethesda, MD, USA). PC3 cells were cultured in RPMI-1640 medium (GibcoBRL, Grand Island, NY, USA) containing an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100μg/ml streptomycin and 0.25 μg/ml amphotericin) (GibcoBRL, Bethesda, MD, USA) and 5% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA, USA) in a humidified incubator (37°C, 5% CO_2 and 95% air). Culture medium was changed every 48 h until the cells were approximately 80% confluent, at which time the cells were dissociated with 0.5% trypsin/ 5.3 mM EDTA in Hank's balanced salt solution (HBSS) (GibcoBRL, Bethesda, MD, USA). The dissociated cells were pelleted by centrifugation at 200 x g for 5 min and then resuspended in RPMI-1640 supplemented with 5% FCS.

Thymidine incorporation assay. To measure the proliferation of PC3 cells, approximately $2x10^4$ cells per well were cultured in 24-well plates (Falcon, Lincoln Park, NJ, USA). After 24 h, PC3 cells were treated with the different concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) for 24 h. The cells were then pulsed with 5 μCi/ml of (^3H) thymidine (NEN Corp., Boston, MA, USA) for 3 h. At the end of this period, the cells were washed twice with phosphate-buffered saline (PBS) and fixed with methanol/acetic acid (3:1). Next, the cells were washed with 1 ml of 0.75 M trichloroacetic acid for 30 sec and then lysed with 0.5 ml of 0.2 N NaOH for 1 h. The cell lysates were then neutralized with an equivalent volume of 0.2 N HCl and transferred to scintillation vials. After the addition of 5 ml of scintillation cocktail (Fisher Scientific, Fair Lawn, NJ, USA), the radioactivities were counted in a β-counter. Amounts of (^3H) thymidine incorporated into DNA were presented as dpm/well.

Doubling-time determination. Growth rates were determined by doubling-time (DT) using Chopra's method (1). Approximately $1x10^4$ viable PC3 cells per well were cultured in 24-well plates (Falcon). After 24 h, PC3 cells were treated with (±)-GP at 0.0, 0.5, 1.0 and 2.0 μM and cell numbers at different treatment time-points after treatment (0, 12, 24, 36, 48, 60 and 72 h) were determined by using a hemacytometer and the trypan blue dye-exclusion method. The trypan blue dye-exclusion method was used to evaluate the cell viability. The cells were examined in a counting chamber under a light microscope. Only viable cells were recorded. The DTs during the exponential growth phase were calculated as $N=N_0 2^n$ where N_0 is the initial population and N is the final population after "n" doublings. The time (g) for the population to be doubled was calculated as $g=t_2-t_0/n$ in which t_2 is the culture

time in hours when N is determined and t_0 is the culture time in hours at which N_0 is determined (1).

Preparation of conditioned media from cultured prostate cancer cells. $2x10^6$ PC3 cells were cultured in 75-cm^2 cell culture flasks containing 10 ml of RPMI-1640 media with 5% FCS. When they attained 80% confluence, they were washed three times with PBS. PC3 cells were treated with 1 μM of (±)- GP and cultured in 10 ml of serum-free RPMI-1640 media with 0.2% bovine serum albumin (BSA) for 24 h. The resultant media were considered to be conditioned media. The media were collected, centrifuged at 2000 x g for 20 min at 4˚C and then filtered through a 0.2 μM filter. The filtrate was stored at -20˚C. The mouse monoclonal anti-TGF-β_1,-β_2,-β_3 antibody (25 μg/ml) was used to block the action of TGFβ_1 produced by conditioned media-treated PC3 cells.

Reverse transcription-polymerase chain reaction (RT-PCR). To determine the effects of (±)-GP on the mRNA expression of some target genes in PC3 cells, PC3 cells were cultured in RPMI-1640 containing 5% FCS in 75-cm^2 flasks at a density of $2x10^6$ viable cells for 24 h. At the end of this culture period, cells were washed twice with RPMI-1640 and cultured in the presence of increasing concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM). The total RNA was isolated from the control cells and (±)-GP-treated cells by TRIzol (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instruction. One μg of RNA was mixed with 5x first strand buffer (GibcoBRL, Bethesda, MD, USA), 0.1 M DDT, 10 mM dNTP, 50 μM random hexamer, 36,100 U/ml RNA guard (Pharmacia Biotech, Piscataway, NJ, USA) and 200 U/μl M-MLV reverse transcriptase (GibcoBRL) in a total volume of 20 μl. Complementary DNA (cDNA) was synthesized by first denaturing at 95˚C for 5 min and then 4˚C for 4 min. The newly synthesized cDNA's were used as templates. Two μl of RT product was mixed with 1.25 μl of $MgCl_2$ (50 mM), 2.5 μl of 10x PCR buffer II, 0.2 μl of Taq polymerase (5 U/μl), and 0.3 μl each of cyclin D_1 5' and 3' primers and 0.3 μl each of β-actin 5' and 3' primers in a total 25 μl. One pair of primers was for amplification of human TGFβ_1 or cyclin D_1 and the other was for human β-actin, which was used as a positive control and loading control. PCR for TGFβ_1 was run for 30 cycles of 95˚C for denaturation for 45 sec, 58˚C for annealing for 45 sec and 72˚C for extension for 1 min. The primer sequences for TGFβ_1 are 5'-CAA GAC CAT CGA CAT GGA GCT GGT GA-3' (sense) and 5'-CAG TTC TCC GTG GAG CTG AAG CA-3' (antisense). PCR for cyclin D_1 was run for 30 cycles of 95˚C for denaturation for 45 sec, 54˚C for annealing for 45 sec and 72˚C for extension for 1 min. The primer sequences for cyclin D1 are 5'-GCT CCT GTG CTG CGA AGT GG-3' (sense) and 5'-TGG AGC CGT CGG TGT AGA TG-3' (antisense). The primer sequences for β-actin are 5'-ACC CAC ACT GTG CCC ATC TAC GA-3' (sense) and 5'-GAT CCA CAT CTG CTG GAA GGT GG-3' (antisense). The final RT-PCR products (10 μl) were run on a 1.5% agarose gel containing ethidium bromide. The specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results are presented as the ratio of TGFβ_1 to β-actin or cyclin D_1 to β-actin

ELISA for TGFβ_1 analysis. To determine the secretion of TGFβ_1 protein, $1x10^4$ PC3 cells were cultured in 24-well culture plates after the cells reached 80% confluence in 75-cm^2 flasks. After 24 h, the PC3 cells were treated with (±)-GP at 0.0, 0.5, 1.0 and 2.0 μM for 24 h. At the end of this treatment period, the conditioned media were collected and TGF-β_1 protein was measured. A commercial antibody sandwich ELISA TGFβ_1 Emax™ ImmunoAssay system (Promega, Madison, WI, USA) was used for this measurement according to the manufacturer's protocol. For ELISA, flat- bottom 96-well plates (Nunc, Kamstrup, Roskilde, Denmark) were coated with 100 μl per well of TGFβ_1 mAb (1 μg/ml) in carbonate coating buffer (0.025 M $NaHCO_3$, 0.0025 M Na_2CO_3, pH 9.74). The plates were sealed with a plate sealer and incubated overnight (18 h) at 4˚C. After removing the contents of each well, plates were blocked with TGFβ block buffer (270 μl per well) for 35 min at 37˚C without shaking. Once incubation had finished, the TGFβ block buffers in wells were removed and experimental samples (conditioned media) were added. The conditioned media collected from (±) - GP-treated and untreated cells were diluted to 1:4 in Dulbecco's phosphate-buffered saline (DPBS) and acidified with 1 μl of 1 N HCl/50 μl of media for 15 min at room temperature and neutralized with 1 μl of 1 N NaOH/50 μl of media. The acidified/neutralized samples (100 μl per well) were added to the well of plates, which were then incubated at room temperature for 90 min with shaking (225 rpm). After washing three times with Tris-HCl-Tween-20 buffer (TBST), the plates were incubated with 100 μl per well of anti-TGFβ_1 pAb (1 μl/ml) in TGFβ_1 Sample 1X buffer for 2 h at room temperature with shaking (225 rpm). After washing three times with TBST, the plates were incubated with 100 μl per well of antibody conjugate in TGFβ sample 1X buffer for 2 h at room temperature with shaking (225 rpm) following by washing three times with TBST. After adding the substrate (mixture of equal volumes of 3,3',5,5'-tetramethylbenzidine [TMB] solution and peroxidase substrate), the plates were incubated at room temperature for 4 min and optical densities were read using a SoftMax (Molecular Devices, Menlo Park, CA, USA) microplate reader at a wavelength of 450 nm. Serial dilutions (0, 15.6, 31.2, 62.5, 125, 250, 500, 1000 pg/ml)

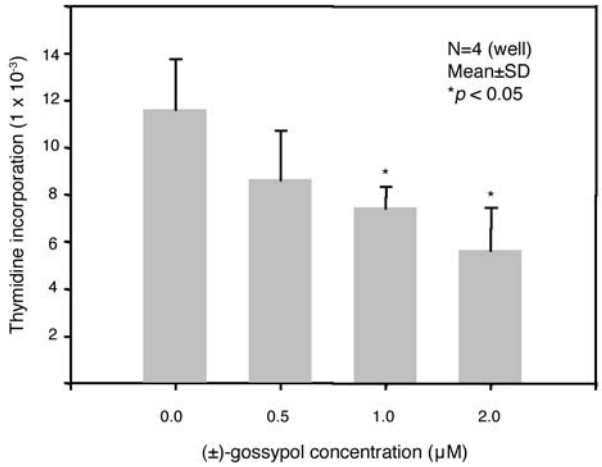

Figure 1. *Effects of (±)-GP on proliferation of PC3 cells. Effects of (±)-GP on proliferation of PC3 cells were assessed by thymidine incorporation assay. PC3 cells were treated with 0.0, 0.5, 1.0 and 2.0 μM of (±)-GP for 24 h. Each bar represents the Mean ± SD of 4 wells. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

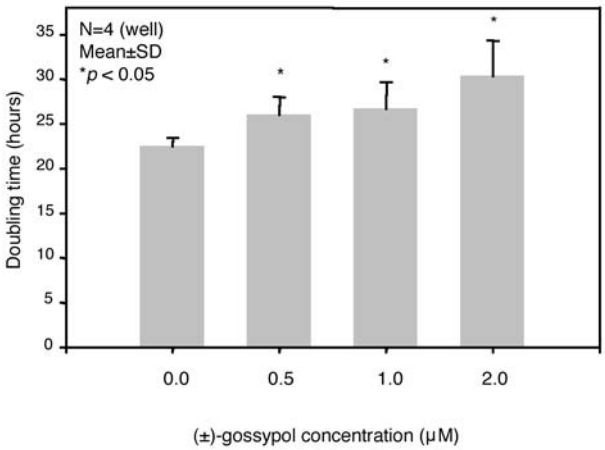

Figure 2. *Effects of (±)-GP on the doubling-time of PC3 cells. Effects of (±)-GP treatment on the growth of PC3 cells were assessed by doubling-time assay. PC3 cells were treated with different concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) for 24 h. Cell numbers were determined using a hemacytometer and the trypan blue dye-exclusion method at different time intervals after treatment (0, 12, 24, 36, 48, 60 and 72 h). Each bar represents the Mean ± SD of 4 wells. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

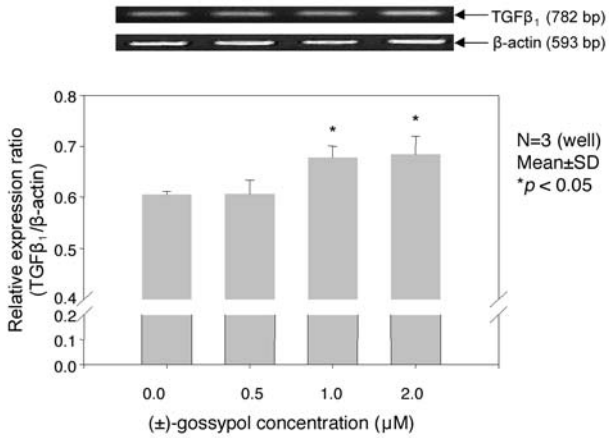

Figure 3. *Effects of (±)-GP on TGFβ_1 mRNA expression in PC3 cells. The TGFβ_1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with (±)-GP at 0.0, 0.5, 1.0 and 2.0 μM for 24 h. Total RNA was isolated from PC3 cells and used for analysis of TGFβ_1 mRNA expression. β-Actin was used as an internal loading control. The results are expressed as the relative expression ratios of TGFβ_1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

of TGFβ_1 standard were used to prepare a standard curve. The TGFβ_1 concentration in the conditioned media is presented as pg/μg cell protein. The total cell protein content in each well was determined by using the Bio-Rad protein micro assay (Bio-Rad Laboratories, Hercules, CA, USA). Cells in each well were lysed by adding 1ml of 0.1 N NaOH. Forty μl of each lysate was diluted 4 times in 0.1 N NaOH and then combined with 40 μl of concentrated Bio-Rad dye binding reagent in a 96-well plate. Optical densities of samples were determined using a SoftMax (Molecular Devices) microplate reader at a wavelength of 595 nm. Serial dilutions (0-50 μl/ml) of bovine serum albumin (Sigma Chemical Co.) were used to prepare a standard curve.

Western blot analysis. After incubation with different concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) for 24 h, PC3 cells were washed twice with ice-cold PBS and then lysed at 4˚C with extraction buffer [20mM Hepes buffer (pH 7.2), 1% Triton-X 100 (v/v), 10% glycerol (v/v), 2 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml leupeptin and 0.5 mM phenylmethlsuphonyl fluoride (PMSF)]. Cell lysates were separated by centrifugation at 15,000 rpm at 4˚C for 30 min. An equal volume of 2x sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% mercaptoethanol) was added to all samples and samples were boiled for 5 min. Forty μg of protein were applied to each well of a 10-well ready Tris-HCl gel (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoreses at 150 voltages for about 1 h, proteins in the ready gel were transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA, USA) by semi-dry transfer system (Bio-Rad) at 80 mAmp constant current for 3 h at room temperature. Protein molecular weight standards obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used for the estimation of molecular size.

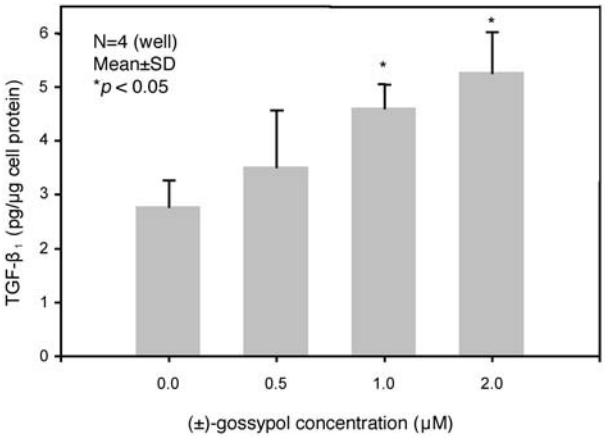

Figure 4. *Effects of (±)-GP on TGFβ_1 secretion in PC3 cells. PC3 cells were treated with different concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) for 24h. At the end of the treatment period, TGFβ_1 secretion was measured by TGFβ_1 Emax^{TM} ImmunoAssay System using acid-activated conditioned media from PC3 cells treated with (±)-GP. Each bar represents the Mean ± SD of 4 wells. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

The membrane containing the transferred proteins was immersed in the blocking buffer (10% milk TBST). The membranes were incubated overnight at 4˚C. Following the blocking procedure, purified mouse anti-human cyclin D_1 gene product monoclonal antibody (1:500) (Santa Cruz Biotechnology)

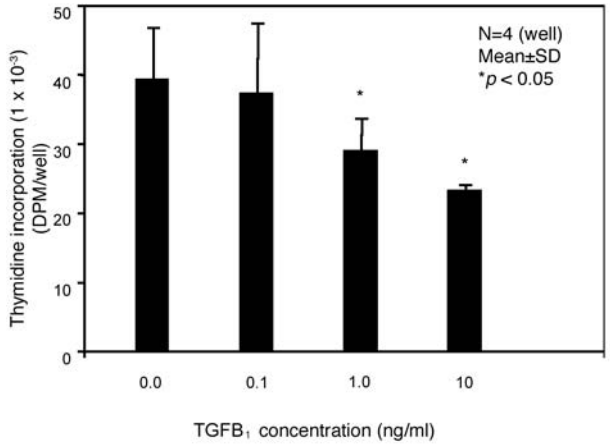

Figure 5. *Effects of TGFβ_1 on proliferation of PC3 cells. Effects of TGFβ_1 on proliferation of PC3 cells were assessed by thymidine incorporation assay. PC3 cells were treated with 0.0, 0.1, 1.0 and 10 ng/ml of TGFβ_1 for 24 h. Each bar represents the Mean ± SD of 4 wells. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

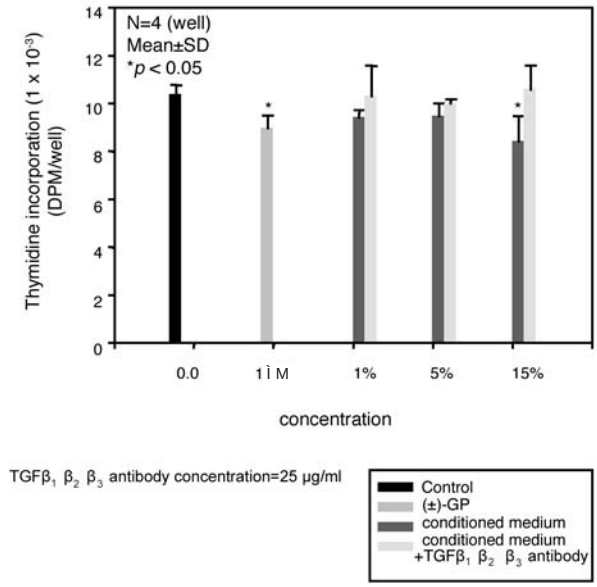

Figure 6. *Effects of conditioned media harvested from (±)-GP-treated PC3 cells on the proliferation of PC3 cells. Effects of 24-hour serum-free conditioned media collected from (±)-GP-treated PC3 cells on the proliferation of PC3 cell line were tested. The details of preparation of conditioned media from PC3 cells was described in Materials and Methods section. Each bar represents the Mean ± SD of 3 experiments. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

in blocking solution was added to the membranes and incubated for 1 h at room temperature. Anti-mouse secondary antibody (Amersham, Piscataway, NJ, USA) was at a concentration of 1:5000 dilutions. After reaction, the membrane was washed and developed by using ECL+Plus Western Blotting Detection system (Amersham, Buckinghamshire, UK) exposed to Hyperfile films (Amersham).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) for 4 culture wells. Minitab statistical software for Windows (Minitab Inc., State College, PA, USA) was used for the statistical analysis. Statistical differences between means were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. A probability (p) of less than 0.05 was considered significant.

Results

Effects of (±)-GP on proliferation in cultured PC3 cells. Prostate cancer during the initial stage of its progression appears to be androgen-dependent, but eventually prostate cancer cells become androgen-independent and refractory to medical therapy. Therefore, the use of chemotherapeutic agents that target the growth of androgen-independent cells has been suggested as a possible effective therapy. In this study, we examined the effects of (±)-GP on the growth of an androgen-independent human prostate cancer cell line, PC3. The effect of (±)-GP on the growth of PC3 cells was determined by thymidine incorporation assay. PC3 cells were treated with increasing concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) for 24 h. The results demonstrated that (±)-GP significantly inhibited PC3 cell growth, as shown in Figure 1. (±)-GP at the concentrations of 0.5, 1.0 and 2.0 μM caused reductions in DNA synthesis by 25.6%, 36.1% and 51.4%, respectively. The (±)-GP decreased the DNA synthesis in PC3 cells in a dose-dependent manner and resulted in a significant reduction in DNA synthesis at the concentrations of 1.0 and 2.0 μM (p<0.05). These results confirm that (±)-GP can inhibit the proliferation of PC3 cells by inhibiting DNA synthesis.

Effects of (±)-GP on the doubling-time of PC3 cells. To understand the effects of (±)-GP on the growth characteristics of PC3 cells, the doubling-times (DT) of PC3 cells treated with different concentrations of (±)-GP (0.0, 0.5, 1.0 and 2.0 μM) were determined. Cell numbers at different treatment time-points (0, 12, 24, 36, 48, 60 and 72 h) were determined by using a hemacytometer and the trypan blue dye-exclusion method. As shown in Figure 2, (±)-GP prolonged the DTs of PC3 cells in a dose-dependent manner. (±)-GP at the concentrations of 0.5, 1.0 and 2.0 μM prolonged DTs of PC3 cell growth by 11.3%, 18.7% and 34.9%, respectively. The results showed that (±) - GP lowers the growth rate of PC3 cells.

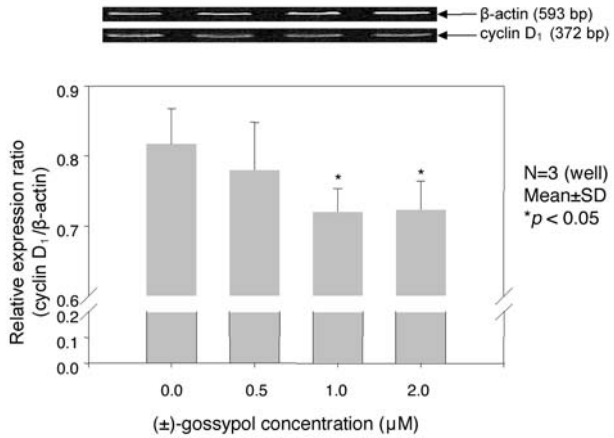

Figure 7. *Effects of (±)-GP on the cyclin D_1 mRNA expression in PC3 cells. The cyclin D_1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with (±)-GP at 0.0, 0.5, 1.0 and 2.0 μM for 24 h. Total RNA was isolated from PC3 cells and used for analysis of cyclin D_1 mRNA expression. β-Actin was used as an internal loading control. The results are expressed as the relative expression ratios of cyclin D_1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represents means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (p<0.05).*

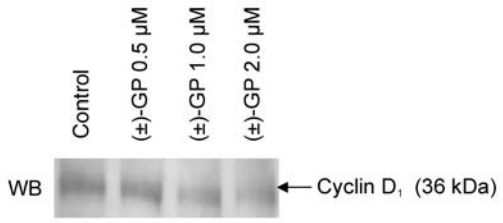

Figure 8. *Effects of (±)-GP on the cyclin D_1 protein expression of PC3 cells. Effects of (±)-GP on the cyclin D_1 protein expression of PC3 cells were measured by Western blot analysis. PC3 cells were treated with (±)-GP at 0.0, 0.5, 1.0 and 2.0 μM for 24 h. Total protein was isolated from PC3 cells and used for cyclin D_1 protein expression. Total proteins were separated on ready gel Tris-HCl gel and transferred onto a PVDF (polyvinylidene difluoride) membrane. Cyclin D_1 protein was detected using mouse anti-human cyclin D_1 gene product monoclonal IgG. In PC3 cells, an approximately 36 kDa protein was detected. The size of this protein corresponds to that of cyclin D_1.*

Effects of (±)-GP on TGFβ_1 mRNA expression and TGFβ_1 protein secretion in cultured PC3 cells. In order to elucidate the mechanism of the inhibitory effects of (±)-GP on cell proliferation, the effects of (±)-GP on TGFβ_1 mRNA expression and protein secretion in PC3 cells was evaluated. RT-PCR results showed that (±)-GP at 1.0 and 2.0 μM resulted in a marked elevation of TGFβ_1 mRNA expression in PC3 cells (Figure 3), while treatment with 0.5 μM (±)-GP had no significant effect on TGFβ_1 mRNA expression. To examine this potential mechanism further, the total amounts of TGFβ_1 protein in the conditioned media were measured by ELISA (Figure 4). TGFβ_1 protein secreted by PC3 cells is in a biologically latent form and can be activated by transient acidification (41). The TGFβ Emax™ immunoassay system can measure only biologically active TGFβ_1 in our assay. Therefore, all measured TGFβ_1 data were generated from acid-activated media. The treatment of (±)-GP significantly increased TGFβ_1 protein secretion in a dose-dependent manner. The (±)-GP at 0.5, 1.0 and 2.0 μM increased TGFβ_1 protein secretions by 1.27-fold, 1.66-fold and 1.90-fold compared to the control, respectively. These results indicate that the inhibitory effects of (±)-GP on the growth of PC3 cells seem to be associated with the induction of TGFβ_1 gene expression and protein secretion.

Effects of TGFβ_1 on the proliferation of PC3 cells. TGFβ_1 is one of the most well known physiological negative regulators of growth of a variety of cells. The inhibitory effects of (±)-GP on PC3 cell growth are associated with increasing TGFβ_1 mRNA expression and TGFβ_1 protein secretion, suggesting TGFβ_1 as a possible mediator of inhibitory effects of (±)-GP. To confirm the ability of PC3 cells to respond to the inhibitory effects of TGFβ_1, PC3 cells were treated with increasing concentrations of TGFβ_1 (0, 0.1, 1 and 10 ng/ml) for 24 h. The effect of TGFβ_1 on proliferation of PC3 cells was assessed by thymidine incorporation assay. As shown in Figure 5, TGFβ_1 inhibited the growth of PC3 cells in a dose-dependent manner. Based on our results, the ability of PC3 cells to produce TGFβ_1 (42) and respond to its inhibitory effects (21) suggests a role for TGFβ_1 as a negative regulator of prostate cancer cells. Therefore, the inhibitory effects of (±)-GP on the prostate cancer cell growth may relate to the augmentation of the inhibitory pathway of TGFβ_1.

Effects of conditioned media harvested from cultured PC3 cells on the proliferation of PC3 cells. In order to determine whether TGFβ_1 produced by PC3 cells was involved in the inhibition of PC3 cell growth, the effect of conditioned media collected from (±)-GP (1 μM)-treated PC3 cells on the proliferation of PC3 cells was examined. We observed that conditioned media collected from (±)-GP-treated PC3 cells at 15% concentration (v/v) led to a significant growth inhibition of PC3 cells (Figure 6). To test whether TGFβ_1 in human prostate cancer PC3 cells mediated the inhibitory effects of (±)-GP, we used the mouse monoclonal anti-TGF-β_1,-β_2,-β_3 antibody to block the endogenous TGFβ_1 effect of conditioned media. As shown in Figure 6, after PC3 cells were incubated for 24 h with mouse monoclonal

anti-TGF-β_1,-β_2,-β_3 antibody (25 μg/ml) and conditioned media, the growth inhibition caused by conditioned media (15%) was completely blocked by anti-TGF-β_1,-β_2,-β_3 antibody (25 μg/ml). These results indicate that TGFβ_1 may be involved in mediating the inhibitory effects of (±)-GP in PC3 cells, and further confirms that TGFβ_1 serves as a potent inhibitor of PC3 cell growth.

Effects of (±)-GP on cyclin D_1 expression in cultured PC3 cells. Previously we have shown that (±)-GP inhibits the growth of human prostate cancer cells by inducing TGFβ_1 gene expression (21). TGFβ_1 is a negative growth regulator that regulates the functions of cyclin D_1 and Rb proteins, which are involved in cell cycle progression (43, 44). Since the antiproliferative effects of (±)-GP on human MCF-7 breast cancer cells is mediated by modulating the expression of Rb and cyclin D_1 protein (22), the mRNA and protein expression of cyclin D_1 in PC3 cells might be affected by (±)-GP treatment. The results showed that (±)-GP at the concentrations of 1.0 and 2.0 μM resulted in a significant decrease in cyclin D_1 mRNA expression and protein expression in PC3 cells in a dose-dependent manner (Figure 7 and Figure 8). Treatment of PC3 cells with 0.5 μM (±)-GP had no effect on the mRNA expression and protein expression of cyclin D_1. The decrease in cyclin D_1 expression parallels the reduction of DNA synthesis in PC3 cells, suggesting that the inhibitory effects of (±)-GP may be mediated by modulating cyclin D_1 expression. Also, we do not exclude that other cell cycle regulators such as p53 protein may be involved in mediating (±)-GP's action (45).

Discussion

(±)-GP is a yellowish polyphenolic pigment that occurs naturally in cottonseed and is also found in cotton plant by-products (46) that are often consumed by humans and food-producing animals. (±)-GP can serve as a potent chemotherapeutic agent against human androgen-dependent and -independent prostate disease. Previous studies from our and other laboratories have shown that (±)-GP can inhibit the growth of human prostate cancer cells (21), human BPH cells (36), human ovarian cancer cells (47), colon cancer cells (48) and human breast cancer cells (49). Furthermore, we have previously reported that 3.0 μM of either (±)-GP or (-)-GP was required to achieve a significant level of growth inhibition of human breast cancer cells (34). In the present study, (±)-GP at a concentration of 1.0 μM significantly inhibited the growth of PC3 cells. Consistent with its ability to inhibit PC3 cell proliferation, our results indicate that (±)-GP inhibited the DNA synthesis of PC3 cells and prolonged DTs of PC3 cells in a dose-dependent manner.

Although our previous research has shown that (±)-GP inhibited 5α reductase activity and 3α-hydroxysteroid dehydrogenase activity in rat testes (35), and induced spermindine/spermine N^1-acetytransferase in canine prostate epithelial cells (50), the mechanism of the inhibitory effect of (±)-GP is not clear. Our previous reports showed that (±)-GP could inhibit cell growth by inducing TGFβ_1 mRNA and blocking the cell cycle at the G_0/G_1-phase in human prostate cancer and human BPH cells (21, 36). Our previous results also showed that the anti-proliferative effect of (±)-GP might be mediated by inducing TGFβ_1 protein production in the stromal cells isolated from human breast adipose tissues (37). The present results showed that (±)-GP treatment markedly elevated TGFβ_1 mRNA expression in PC3 cells and stimulated TGFβ_1 secretion of PC3 cells after 24-hour incubation. Furthermore, the effect of (±)-GP in enhancing the secretion of TGFβ1 protein correlates with its inhibitory effects on DNA synthesis and growth rate of PC3 cells. As TGFβ1 affects cell cycle-regulating proteins, such as cyclin D_1 and Rb proteins, which are involved in cell cycle progression from G_1-phase to S-phase, this finding suggests that TGFβ_1 is a potential physiological regulator of normal prostate cells, cancer cells and human breast cancer cells. It is known that (±)-GP induced cell cycle arrest at G_1/S-phase by decreasing Rb protein expression, Rb protein phosphorylation and cycle D_1 protein expression in MCF-7 cells (22). It has also been reported that TGFβ_1 treatment reduced cyclin D_1 mRNA and protein expression in rat intestinal epithelial cells (51) and the expression of Rb1 mRNA and Rb protein phosphorylation in Mv1Lu cells (52). These findings led us to test whether (±)-GP could affect the mRNA and protein expression of cyclin D_1 in PC3 cells. The results of our experiment demonstrate that (±)-GP treatment decreased cyclin D_1 mRNA expression and protein expression in PC3 cells. Our findings suggest that the anti-proliferative effects of (±)-GP are mediated by inducing TGFβ_1 gene expression, which further regulates the involvement of cyclin D_1 protein in cell cycle progression. The (±)-GP probably exerts its effect at the transcriptional level, either by increasing transcription or by modifying the stability of TGFβ_1 mRNA within the cell cycle regulatory pathway (53). These findings suggest that (±)-GP may have potential to become chemopreventive and chemotherapeutic agents against human prostate cancer.

TGFβ_1 is an important growth inhibitor of a variety of cancer cells (44, 54). Under our experimental conditions, our results showed that TGFβ_1 is able to significantly inhibit the growth of human prostate cancer PC3 cells in a dose-dependent manner. TGFβ_1 at 1.0 ng/ml significantly decreased DNA synthesis in PC3 cells by 26.2% compared with the control group. The results that PC3 cells secreted and responded to TGFβ_1 suggest that TGFβ_1 can function

as a negative autocrine growth regulator for PC3 cells. We have demonstrated that the addition of 15% conditioned media significantly inhibited the proliferation of PC3 cells by 18.9% compared with the control group. When anti-TGFβ_1-β_2-β_3 antibody at 25 μg/ml was added to the conditioned media, the growth inhibition of PC3 cells induced by 15% conditioned media was completely reversed. These results, along with the observations that (±)-GP significantly increased TGFβ_1 secretion and TGFβ_1 gene expression, strongly support the hypothesis that the anti-proliferative activity of (±)-GP is mediated by TGFβ_1 secretion in PC3 cells.

Experimental results have shown that, in addition to the *in vitro* anticancer effects of (±)-GP, (±)-GP also suppressed the *in vivo* growth of Ehrlich ascites tumor cells hosted in NMRI mice (55) and MAT-Lyle cells transplanted in Copenhagen rats, and prolonged the survival of mice implanted with mouse mammary carcinoma 755 cells (23). (±)-GP caused a reduction in the lung and lymph node metastasis of MAT-LyLu-bearing Copenhagen rats and also caused a decrease in the invasive ability of MAT-LyLu cells *in vitro* (20, 56). In a chronic oral trial in man, (±)-GP did not result in myelosuppression (57). Furthermore, (±)-GP caused tumor regression in advanced cancer patient with gliomas (58), adrenal cell carcinoma (28) and breast cancer (59) that was refractory to standard therapy. (-)-GP, an enantiomer of (±)-GP, was more potent than cisplatin, melphalan and dacarbazine in the melanoma lines, and cisplatin and dacarbazine in lung cancer lines (60). These results have indicated that (±)-GP have a potential value as chemopreventive agents against prostate cancer.

Although androgen ablation is a primary method for prostate cancer treatment, the response of patients is temporary. This transient response to androgen withdrawal is due to transition from androgen-dependent cancer cells to androgen-independent cancer cells (7, 61). While androgen-responsive prostate cancer cell death is induced following withdrawal, androgen-independent prostate cancer cells restore tumor growth. An agent that targets androgen-independent cancer cells combined with androgen-ablation could be clinically useful in the treatment of prostate cancer (7). Our results indicated that (±)-GP are potent inhibitors of androgen–independent prostate cancer cells. In addition, the ability of (±)-GP to inhibit 5-α reductase within the testes (35) suggests effectiveness against androgen–dependent prostate cancers. Thus, (±)-GP could be a potent chemopreventive agents against androgen-dependent and –independent prostate cancer cells.

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