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UMI
THE REGULATION OF PROSTATE CELL GROWTH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

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* * * * *

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ABSTRACT

Prostatic cancer and benign prostatic hyperplasia (BPH) are the common prostate diseases found in American men over 50 years old. We evaluated the chemotherapeutic efficacy of racemic gossypol [GP (±)], an antifertility agent, on prostate cancer and the role of estrogen on canine BPH. GP (±) was shown to inhibit growth and metastasis of prostate cancer cells in the in vitro system. Effect of GP (±) on the growth of prostatic cancer cells may be mediated by inducing production of transforming growth factor-β1 (TGFβ1) in prostate cancer cells. TGFβ1, in turn, can regulate the expression of cell cycle regulatory protein cyclin D1 that regulate cell cycle progression of prostate cancer cells. Gossypol (-) [GP (-)] and cottonseed oil, like GP (±), also exhibit similar anti-proliferative effects on the prostatic cancer cells. Moreover, the inhibitory dose of GP (-) and cottonseed oil have a greater potency than that of GP (±). GP (±) was also shown to inhibit the O$_2$ consumption and CO$_2$ production in prostate cancer cells. This inhibition may relate to inhibiting mitochondrial succinic dehydrogenase activity in those cells.

Recently, we isolated a novel subline of the MAT-LyLu cells isolated from metastasized lungs of MAT-LyLu bearing Copenhagen rats (MLL cell). MLL cells displayed more invasive properties than MAT-LyLu cell, while MLL cells possessed lower mRNA levels of nm23, a metastasis suppressor gene, than MAT-LyLu cells. Both MLL and MAT-LyLu cells were susceptible to GP (±), which induced a dose-dependent
inhibition of invasive activity with increasing the expression of nm23 mRNA. The invasive inhibition of GP (±) on MLL and MAT-LyLu cells might be mediated by elevating the expression of nm23 metastasis suppressor gene.

Experimentally, estrogens are associated with human BPH. The increase in the proliferation of canine prostatic stromal cells and the decrease in the estrogen receptor beta (ERβ) mRNA expression of canine prostatic cells with ages were observed. The age-dependent and cell-specific difference in ERβ mRNA expression of canine prostate may contribute to the proliferative imbalance between epithelial/stromal cells of aging dogs, associated with the development of canine BPH. Also, the response of canine prostatic cells to 17β-estradiol (E2) treatment is cell-specific and age-dependent.
Dedicated to my parents, my wife and my daughter
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Gossypol [(±)] is a naturally occurring polyphenolic yellow pigment present in cottonseed products. Natural GP (±) exists as a racemic mixture of GP (+) and GP (-) enantiomers and has been shown to be powerful inhibitor of tumor growth. GP (±) inhibits in vitro growth of Dunning rodent prostate cancer cells (MAT-LyLu), human prostate cancer cells derived from a bone marrow metastasis (PC3), MCF-7 and primary cultures of human prostate cells, as well as the in vivo tumor growth and lung and lymph node metastasis of the androgen-independent prostate cancer cell line, MAT-LyLu, after implantation into Copenhagen rats. In the present study, we used three prostate cancer cell lines (MAT-LyLu, PC3 and LNCaP) to examine the effects of GP (±) on the proliferation of prostate cancer cells. Because the anti-proliferative effects of GP (±) could be exerted mainly by either GP (-) or GP (+), the anti-proliferative effects of GP (±), GP (+), and GP (-) on prostate cancer cells were investigated. The proliferation of
prostate cancer cells was determined by thymidine incorporation assay and doubling time (DT) determination. The mechanisms of action of GP (±) on the proliferation of prostate cancer cells were determined by RT-PCR analysis and ELISA assays. The results showed that GP (±) significantly inhibited the proliferation of three prostate cancer cell lines in a dose-dependent manner. GP (±) concentrations of 0.25, 1.0, and 4.0 μM were required to achieve a significant growth inhibition in MAT-LyLu, PC3, and LNCaP cells, respectively (P<0.05). Similarly, GP (±) prolonged the DT of MAT-LyLu cells at 0.25 μM and PC3 cells at 1.0 μM respectively (P<0.05). In the study comprising the effects of GP (±), GP (+) and GP (-) enantiomers on the proliferation of PC3 cells, GP (-) induced significant growth inhibition at 1.0 μM and was shown to be more potent than GP (±). However, GP (+) had no effect on the proliferation of PC3 cells. We have also shown that PC3 cell proliferation is significantly suppressed by treatment with 5% refined cottonseed oil containing 0.45 ppm [0.78 μM GP (±)]. The cottonseed oil exhibited an inhibitory dose that represents a greater potency than that of GP (±).

The mechanisms of action of GP (±) and its enantiomers on the proliferation of prostate cancer cells were investigated by RT-PCR analysis and ELISA assays. The expression of transforming growth factor beta1 (TGFβ1), cyclin D1 and retinoblastoma susceptibility gene (Rb1) mRNA in three prostate cancer cell lines after treatment of GP (±) were determined by RT-PCR analysis. TGFβ1 mRNA was expressed in PC3 and MAT-LyLu cells, but was absent in LNCaP cells. RT-PCR results showed that GP (±) elevated the mRNA expression of TGFβ1 in MAT-LyLu and PC3 cells. Refined cottonseed oil also increased the mRNA expression of TGFβ1 in PC3 cells. Consistent with these findings, the ELISA assay showed that GP (±) stimulated TGFβ1 secretion in
PC3 cells as well MAT-LyLu cells after 48-hour incubation. We also found that exogenous TGFβ1 significantly inhibited the growth of MAT-LyLu and PC3 cells in a dose-dependent manner. 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 monoclonal mouse anti-TGFβ1,-β2,-β3 antibody. Thus, this study indicates that the inhibitory effects of GP (±) and cottonseed oil on the proliferation of androgen-independent prostate cancer cells may be mediated by increased the TGFβ1 production. In addition, we also investigated other important cell cycle regulatory genes (cyclin D1 and Rb1) for their role in mediating the effects of GP (±) on the growth of prostate cancer cells. It is known that cyclin D1 and Rb protein are involved in mediating the action of GP (±) in the MCF-7 human breast cancer cell line. Our results showed that GP (±), GP (-) and refined cottonseed oil might cause the reduction of cyclin D1 mRNA expression in PC3 cells, while a higher dose of GP (±) (8.0 µM) might cause the same change in LNCaP cells. In contrast, GP (±) had no effect on the Rb1 mRNA expression in PC3 cells. These results indicate that inhibitory effects of GP (±) and refined cottonseed oil on cellular proliferation of human prostate cancer are associated with induction of TGFβ1, which in turn influence the expression of the cell cycle-regulatory protein, cyclin D1, in prostate cancer cells.

**Introduction**

Prostate cancer is the most common malignancy in men and is the second leading cause of male cancer death in the US (Chopra et al., 1997; Parker et al., 1997). Previous research has shown that androgens such as testosterone and dihydrotestosterone stimulate
the growth of this malignancy and are involved in prostate cancer pathogenesis (Kallio et al., 1996; Wilding et al., 1995). The mechanism of prostatic carcinogenesis and tumorigenesis likely involves a multistep progression from precancerous cells to cells which are proliferative and metastasized (Berges et al., 1995). The growth and development of prostate cancer cells appear to be androgen-dependent initially (Huggins et al., 1941), with the androgens acting through their receptors [androgen receptor (AR)] to regulate the transcription of downstream genes controlling the cellular growth and differentiation (Isaacs et al., 1993). Androgen deprivation and antiandrogens inhibit the AR’s transcription function, thus, suppressing its ability to act as a transcription factor. This results in blockade of the survival signal elicited by androgens and the subsequent induction of apoptosis (Nessler-Menardi et al., 2000; Kyprianou et al., 1990). Therefore, androgen deprivation is the primary treatment method for prostate cancer (Isaacs et al., 1993; Scott et al., 1980). 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 (Isaacs et al., 1993; Nessler-Menardi et al., 2000).

Gossypol (±) [GP (±)], is a naturally occurring yellow pigment present in cottonseed products (cottonseed oil and cottonseed meal) consumed by humans and food-producing animals (Boatner et al., 1949). Natural GP (±) exists as a racemic mixture of (+) and (-) enantiomers (Joseph et al., 1986). The axial asymmetry of the GP (±) molecule results in two optical isoforms, GP (-) and GP (+). It is generally believed that GP (±) is biologically active in its free form and less active in its
bound form (Jones et al., 1991). 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 (Lin et al., 1980; Lin et al., 1987a; Ranga et al., 1990) and females (Gu et al., 1990; Gu et al., 1991; Lin et al., 1985; Lin et al., 1989). 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 (National Institutes of Health., 1991).

Research results have shown that GP (±) inhibits the proliferation of many human cancer cells in vitro and in vivo (Chang et al., 1993; Shidaifat et al., 1996; Ligueros et al., 1997). A significant body of evidence indicates GP's anticancer and antiproliferative effects on a variety of human cancer cell lines including those of the breast, prostate, ovary, cervix, uterus, adrenals, pancreas and colon (Rao et al., 1985; Band et al., 1989; Benz et al., 1988; Thomas et al., 1991; Stein et al., 1992; Flack et al., 1993; Coyle et al., 1994; Liang et al., 1995). 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 rats (Chang et al., 1993). 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 (Joseph et al., 1986), human reproductive tumor cell line (Band et al., 1989), melanoma cell line (Blackstaffe et al., 1997), and breast cancer cell lines (Benz et al., 1990; Liu et al., 2002) and ovarian cancer cell lines (Band et al., 1989).
Although the precise mechanism of action of GP (±) is still unknown, GP (±) has been shown to inhibit some enzymes involved in steroidogenesis such as 5α-reductase and 3α-hydroxysteroid dehydrogenase (Moh et al., 1993). Our results demonstrate that GP (±) acts via a not-yet-defined mechanism to exert its antiproliferative and antimetastatic effects on prostate cancer cells. GP (±) arrests the cell cycle of androgen-independent human prostate cancer cell lines (PC3) and primary cultured cells isolated from benign prostatic hyperplasia (BPH) tissue and human breast tissue in association with increases in the expression of transforming growth factor β1 (TGFβ1) mRNA (Shidaifat et al., 1996; Shidaifat et al., 1997; Zhang et al., 1998) suggesting the involvement of TGFβ1 in GP (±)-induced growth inhibition. TGFβ1 is a potent inhibitor of epithelial cell growth and has been shown to mediate the anti-proliferative effects of many antitumor agents such as vitamin D₃ (Koil et al., 1995) and tamoxifen (Benson et al., 1996). TGF β₁ exerts its effects by binding to a cell surface receptor and triggering a signaling pathway that regulates the factors involved in the cell cycle such as Rb, cyclin and cyclin-dependent kinase (Yingling et al., 1995).

In this study, we used three prostate cancer cell lines: PC3, MAT-LyLu and LNCaP. The PC3 cell line was established from organ metastases of prostate adenocarcinoma and its proliferation is androgen-independent (Kaighn et al., 1978). The MAT-LyLu cell line was derived originally from the Dunning R3327 H prostate cancer cell line, which is androgen-dependent, slow-growing, and non-metastatic. After 20 years of in vivo selection, the MAT-LyLu cell line became fast-growing, androgen-independent, and locally invasive (Isaacs et al., 1981). The LNCaP cell line was established from a lymph node metastasis of prostate adenocarcinoma, and its
proliferation is androgen-dependent (Horoszewicz et al., 1983). The objective of this study was to investigate and compare anti-proliferative activities and potential mechanisms of GP (±) in prostate cancer cell lines. In addition, the comparative effects of GP (±), GP (+), and GP (-) on the growth of androgen-independent prostate cancer cell line, PC3, was explored. Cottonseed oil, which contains GP (±) in very small quantities, is present in many foods, including salad dressing, shortening, margarine, some canned and snack foods and in abundant quantities in dietary replacements. Since GP (±) has been shown to be a powerful inhibitor of tumor growth, we have also attempted to investigate the anti-proliferative effects of cottonseed oil in PC3 cells.

Materials and methods

Reagent

GP (±), GP (-) and GP (+) were the generous gifts from Dr. Michael Dowd, U.S.D.A. Southern Regional Research Center (New Orleans, LA). GP (±), GP (+), GP (-) were dissolved in 100% ethanol to make 10 mM stock solutions. The solutions were aliquoted into glass vials and stored at -20°C. Treatment solutions were prepared by the dilution of stock solution in culture medium. Ultrapure natural human TGFβ1 and monoclonal mouse anti-TGF-β1,-β2,-β3 antibody were purchased from Genzyme Corp. (Cambridge, MA). The refined cottonseed oil used for this study was supplied by Dr. Peter Wan of the U.S.D.A. Southern Regional Research Center (New Orleans, LA) and contained a minimally detectable level of GP at 9 ppm which is about 1/50 of the FDA’s allowable limit (450 ppm). Because 9 ppm of GP (±) in refined cottonseed oil is approximately equal to be 15.5 µM, 1%, 5%, and 25% refined cottonseed oil are equal to
0.155 μM, 0.78 μM, and 3.8687 μM respectively. Corn oil was purchased from Kroger Food Store (Columbus, OH).

**Cell culture**

The human prostate cancer cell lines, PC3, and LNCaP, were originally obtained from American Type Tissue Culture Collection (Bethesda, MD). MAT-LyLu cells, was the generous gift of Drs. J.R. Drago and P. Ghosh of the Department of Surgery, College of Medicine, The Ohio State University, who originally obtained the cells through the courtesy of Dr. J.T. Isaacs, Johns Hopkins University. Cells were cultured in RPMI-1640 medium (GibcoBRL, Grand Island, NY) containing an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100μg/ml streptomycin, and 0.25 μg/ml amphotericin) (GibcoBRL, Bethesda, MD) and 5% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA) in a humidified incubator (37°C, 5% CO₂ 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). The dissociated cells were pelleted by centrifugation at 200 xg for 5 min and then resuspended in RPMI-1640 supplemented with 5% FCS.

**Thymidine incorporation assay**

To measure the cell proliferation of PC3 cells, MAT-LyLu cells or LNCaP cells, approximately 2x10⁴ cells per well were cultured in 24–well plates (Falcon, Lincoln Park, NJ). After 24 h, PC3 cells were treated with the different concentrations of GP (±) (0.0,
0.5, 1.0 and 2.0 μM), with the different concentrations of refined cottonseed oil [1% {0.15 μM GP (±)}, 5% {0.78 μM GP (±)} and 25% {3.86 μM GP (±)}] or with the different concentrations of corn oil (1%, 5% and 25%) respectively. Similarly, after 24 h, MAT-LyLu cells and LNCaP cells were treated with the different concentrations of GP (±) at 0.0, 0.25, 0.5, and 1.0 μM and at 0.0, 2.0, 4.0 and 8.0 μM for 24 h respectively. The cells were then pulsed with 5 μCi/ml of (3H) thymidine (NEN Corp., Boston, MA) for 3 h. At the end of this period, the cells were washed twice with HBSS and fixed with methanol/acetic acid (3:1). Next, the cells were washed with 1ml of 0.75 M trichloroacetic acid for 30s 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), the radioactivities were counted on a β-counter. Amounts of (3H) thymidine incorporated into DNA are presented as dpm/well.

Doubling time analysis

Growth rates were determined by doubling time (DT) using Chopra’s method (Chopra et al., 1996). Approximately 1x10⁴ viable PC3 cells or MAT-LyLu 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 MAT-LyLu cells were treated with GP (±) at 0.0, 0.25, 0.5 and 1.0 μM respectively. Cell numbers at different times after treatment (0, 12, 24, 36, 48, 60, and 72 h) were determined 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 with a light microscope.
Only viable cells were recorded. The DTs during the exponential growth phase were determined 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 double 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 (Chopra et al., 1996).

**Preparation of conditioned media from prostate cancer cells**

2x10⁶ PC3 cells were cultured in 75-cm² cell culture flasks containing 10 ml 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 serum-free RPMI-1640 media with 0.2% bovine serum albumin (BSA) for 24 h. The resultant media were considered to be conditioned media. Media were collected, centrifuged at 2000 g for 20 min at 4°C, and then filtered through a 0.2 μM filter. The filtrate was stored at -20°C. PC3 cells were cultured in the presence of conditioned media [1% (7.03 pg/ml), 5% (35.13 pg/ml), and 15% (105.4 pg/ml)] for 24 h. The monoclonal mouse anti-TGF-β1, β2, β3 antibody (25 μg/ml) was used to block the action of TGFβ1 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 PC3 cells or MAT-LyLu cells or LNCaP cells, PC3 cells or MAT-LyLu cells or LNCaP cells were cultured in RPMI-1640 containing 5% FCS in 75 cm² flasks at a density of 2x10⁶ viable cells for 24 h. At the end of this culture period, PC3 cells or MAT-LyLu cells or LNCaP
cells were washed twice with RPMI-1640 and cultured in the RPMI-1640 medium containing different concentrations of GP (±) for 24 h. PC3 cells and MAT-LyLu cells were treated with different concentrations of GP (0.0, 0.5, 1.0 and 2.0 μM) and (0.0, 0.25, 0.5, and 1.0 μM) for 24 h respectively, which LNCaP cells were treated with different concentrations of GP (0.0, 2.0, 4.0 and 8.0 μM) for 24 h. Similarly, PC3 cells were treated with 1%, 5% and 25% of refined cottonseed oil or 1%, 5% and 25% of corn oil for 24 h. The total RNA was isolated from the control cells and treated cells by TRIzol (Invitrogen Co., Carlsbad, CA) according to instruction of manufacture. 1 μg of RNA was mixed with 5x first strand buffer (GibcoBRL, Bethesda, MD), 0.1 M DDT, 10 mM dNTP, 50 μM random hexamer, 36,100 U/ml RNA guard (Pharmacia Biotech, Piscataway, NJ), 200 U/μl M-MLV reverse transcriptase (GibcoBRL, Bethesda, MD) 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. 2 μl of RT product was mixed with 1.25 μl of MgCl₂ (50 mM), 2.5 μl of 10x PCR buffer II, 0.2 μl of Tag polymerase (5 U/μl), and 0.3 μl each of cyclin D1 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 TGFβ1 or cyclin D1, or Rb1, and other was for human β-actin which used as a positive control and loading control. PCR for TGFβ1 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 TGFβ1 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 D1 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). PCR for Rbl was run for 30 cycles
of 95°C for denaturation for 45 sec, 50°C for annealing for 45 sec and 72°C for extension
for 1 min. The primer sequences for Rbl are 5'-CTC TCG TCA GGC TTG AGT TT-3'
(sense) and 5'-ATG GAC ACT GAT TTC TAT GT-3' (antisense). The primer sequences
for β-actin are 5'-ACC CAC ACT GTG CCC ATC TAG 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). The results
are presented as the ratio of TGFβ1 to β-actin or cyclin D1 to β-actin or Rbl to β-actin.

ELISA for TGFβ1 analysis

To determine the secretion of TGFβ1 protein, 1x10⁴ PC3 cells and MAT-LyLu
cells were cultured in 24-well culture plates after the cells reach 80% confluence in 75-
cm² flasks. After 24 h, the PC3 cells and MAT-LyLu cells were treated with GP (±) at
0.0, 0.5, 1.0, and 2.0 µM for 24 h respectively. At the end of this treatment period, the
conditional media were collected and TGF-β1 protein was measured. A commercial
antibody sandwich ELISA TGFβ1 Emax™ ImmunoAssay system (Promega, Madison,
WI) 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 TGFβ1 mAb (1 µg/ml) in carbonate coating buffer (0.025 M
NaHCO₃, 0.0025 M Na₂CO₃, 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 finished, the TGFβ block buffers of wells were removed and experimental sample (conditioned media) added. The conditioned media collected from GP (±) treated and untreated cells were diluted 1:4 in 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-TGFβ1 pAb (1 μl/ml) in TGFβ1 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 in room temperature for 4 min and optical densities were read using a SoftMax (Molecular Devices, Menlo Park, CA) microplate reader at a wavelength of 450 nm. Serial dilutions (0, 15.6, 31.2, 62.5, 125, 250, 500, 1000 pg/ml) of TGFβ1 standard were used to prepare a standard curve. The TGFβ1 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 microassay (Bio-Rad Laboratories, Hercules, CA). Cells in each well were lysed by adding 1ml of 0.1 N NaOH. 40 μl of each lysate was diluted 4 times in 0.1 N NaOH and then combined with 40 μl of concentration 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., St. Louis, MO) were used to prepare a standard curve.

**Western Blot Analysis**

After incubated with different concentrations of GP (±) 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 phenylmethlysuphonyl fluoride (PMSF)]. Cell lysates were separated by centrifugation at 15,000 rpm at 4°C for 30 min. Added an equal volume of 2x sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% mercaptoethanol) to all samples and samples were boiled for 5 min. Applied equal amounts of protein in each lane (40 µg of protein for each lane) to each well of 10-well ready gel Tris-HCl gel (Bio-Rad Laboratories, Richmond, CA). After electrophoreses at 150 voltage for about 1 h. Proteins in the ready gel were transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Bedford, MA) by semi-dry transfer system (Bio-Rad, Hercules, CA) at 80 mAmp constant current for 3 h at room temperature. Protein molecular weight standards obtained from Santa Cruz Biotechnology were used for the estimation of molecular size.

The membrane containing the transferred proteins was immersed in the blocking buffer (10% milk PBST). The membranes were incubated overnight at 4°C. Following
the blocking procedure, Purified mouse anti-human cyclin D1 gene product monoclonal antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution was added to the membranes and incubated for 1 h at room temperature. Anti-mouse secondary antibody (Amersham, Piscataway, NJ) was at a concentration of 1:5000 dilution. After reaction, membrane was washed and developed by a chemiluminescence ECL+Plus Western Blotting Detection system (Amersham, Buckinghamshire, UK) exposed to Hyperfile (Amersham, Buckinghamshire, UK).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) for 4 cultures well. Using Minitab statistical software for Windows (Minitab Inc., State College, PA) performed 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 the proliferation of 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 show that GP (±) significantly inhibits PC3 cell growth as shown in Figure 1.1. GP (±) at the concentration of 0.5, 1.0, and 2.0 μM caused reductions in DNA synthesis by 19.1%, 62.4%, and 73.5% respectively. GP (±) decreased the DNA synthesis in PC3 cells in a dose-dependent manner and resulted in a significant reduction in DNA synthesis at the concentration 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 times after treatment (0, 12, 24, 36, 48, 60, and 72 h) were determined using a hemacytometer and the trypan blue dye-exclusion method. As shown in Figure 1.2, GP (±) prolonged the DTs of PC3 cells in a dose-dependent manner. GP (±) at the concentration of 0.5, 1.0 and 2.0 μM prolongs DTs of PC3 cell growth by 11.3%, 18.7% and 34.9% respectively. The results show that GP (±) could slows the growth rate of PC3 cells.

**Effect of GP (±) on the TGFβ1 mRNA expression and TGFβ1 secretion of cultured PC3 cells.**

In order to elucidate the mechanism of the inhibitory effects of GP (±) on cell
proliferation, the effects of GP (±) on TGFβ1 mRNA expression and 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 TGFβ1 mRNA expression in PC3 cells (Figure 1.3), while the treatment with 0.5 μM GP (±) had no significant effect on TGFβ1 mRNA. To examine this potential mechanism further, the total amounts of TGFβ1 protein in the conditioned media were measured by ELISA (Figure 1.4). TGFβ1 protein secreted by PC3 cells is in a biologically latent form and can be activated by transient acidification (Lawrence et al., 1985). The TGFβ Emax™ immunoassay system can measure only biologically active TGFβ1 in our assay. Therefore, all data were generated from acid-activated media. GP (±) treatment significantly increased TGFβ1 secretion in a dose dependent manner. GP (±) at 0.5, 1.0, and 2.0 μM increased TGFβ1 secretions by 1.27 fold, 1.66 fold, and 1.90 fold compared to the control, respectively. This result indicates that the inhibitory effects of GP (±) on the growth of PC3 cells seem to be associated with the induction of TGFβ1 gene expression.

*Effects of TGFβ1 on the proliferation of PC3 cells*

TGFβ1 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 TGFβ1 mRNA expression and TGFβ1 protein secretion, suggesting TGFβ1 as a possible mediator of inhibitory effects of GP (±) on PC3 cell growth. To confirm the ability of PC3 cells to respond to the inhibitory effects of TGFβ1, PC 3 cells were treated with increasing concentrations of TGFβ1 (0, 0.1, 1, and 10 ng/ml) for 24 h. The effect of TGFβ1 on proliferation of PC3 cells was assessed by thymidine
incorporation assay. As shown in Figure 1.5, TGFβ1 inhibited the growth of PC3 cells in a dose-dependent manner. Based on our results, the ability of PC3 cell to produce TGFβ1 (Derynck et al, 1987; Ikeda et al, 1987) and respond to its inhibitory effects (Shidaifat F., 1997) suggests a role for TGFβ1 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 TGFβ1.

Effects of conditioned media harvested from cultured PC3 cells on the proliferation of PC3 cells.

In order to determine whether TGFβ1 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 led to a significant growth inhibition of PC3 cells (Figure 1.6). From our ELISA results described in previous section, the amount of TGFβ1 present in 1ml of 15%-conditioned media is equal to about 105.4 pg of TGFβ1 (105.4 pg/ml). Similarly, the amount of TGFβ1 secretion in 1 ml of 1%-conditioned media and 5%-conditioned media are equal to about 7.03 pg and 35.13 pg of TGFβ1 respectively. In order to test whether TGFβ1 in human prostate cancer PC3 cells mediated the inhibitory effects of GP (±), we used the monoclonal mouse anti-TGF-β1,-β2,-β3 antibody to block the endogenous TGFβ1 effect of conditioned media. As shown in Figure 1.6, after PC3 cells were incubated for 24 h with monoclonal mouse anti-TGF-β1,-β2,-β3 antibody (25 μg/ml) and conditioned media, the growth inhibition caused by conditioned media (15%) was completely
blocked by anti-TGF-β1,-β2,-β3 antibody (25 μg/ml). This result indicates that TGFβ1 may be involved in mediating the inhibitory effects of GP (±) in PC3 cells, and further confirms that TGFβ1 serves as a potent inhibitor of PC3 cell growth.

Effects of GP (±) on the proliferation of MAT-LyLu cells

In order to test whether GP (±) possess the same anti-proliferative property in different androgen-independent prostate cancer cells, we examined the effects of GP (±) on the growth of MAT-LyLu cells. The effect of GP (±) on the growth of MAT-LyLu cells was determined by thymidine incorporation assay. MAT-LyLu cells were treated with increasing concentrations of GP (±) (0.0, 0.25, 0.5, and 1.0 μM) for 24 h. The results showed that GP (±) significantly inhibited MAT-LyLu cell growth as shown in Figure 1.7. GP (±) at the concentrations of 0.25, 0.5, and 1.0 μM caused reductions in DNA synthesis by 33.4%, 53.2%, and 94.3% respectively. GP (±) decreased the DNA synthesis in MAT-LyLu cells in a dose-dependent manner and resulted in a significant reduction in DNA synthesis at the concentration of 0.25, 0.5, and 1.0 μM (P<0.05). These results confirm that GP (±) can inhibit the proliferation of MAT-LyLu cells by inhibiting DNA synthesis, and is consistent with effects of GP (±) on the proliferation of PC3 cells. The results showed that MAT-LyLu cells were more sensitive to GP (±) treatment than PC3 cells.

Effects of GP (±) on the doubling time of MAT-LyLu cells

We assessed the anti-proliferative effects of GP (±) on the growth of MAT-LyLu cells by doubling time analysis. MAT-LyLu cells were treated with different
concentrations of GP (±) (0.0, 0.25, 0.5, and 1.0 μM) for 24 h and then cell numbers were counted using a hemacytometer and the trypan blue dye exclusion method at different times after treatment (0, 12, 24, 36, 48, 60, and 72 h). As shown in Figure 1.8, GP (±) at the concentrations of 0.25, 0.5 and 1.0 μM prolonged DTs of MAT-LyLu cell growth by 33%, 42.7% and 89.2% respectively (P<0.05). The results showed that GP (±) could slow the growth rate of MAT-LyLu cells, and that MAT-LyLu cells are more sensitive than PC3 cells to GP (±)’ inhibitory actions. The differences between PC3 cells and MAT-LyLu cells in both thymidine incorporation and DT values are probably due to differences in the growth rate between the two cell types in culture.

**Effect of GP (±) on the TGFβ1 mRNA expression and TGFβ1 secretion of cultured MAT-LyLu cells.**

The mechanism by which GP (±) triggers an inhibitory pathway leading to growth inhibition of cultured human prostate cancer PC3 cells and human BPH epithelial cells at the G0/G1 phase of the cell cycle is known (Shidaifat et al., 1996; Shidaifat et al., 1997). In order to elucidate the mechanism of inhibitory effects of GP (±) on the proliferation of MAT-LyLu cells, the effects of GP (±) on TGFβ1 mRNA expression and secretion in MAT-LyLu cells were measured. RT-PCR results showed that GP (±) at 0.5 and 1.0 μM resulted in a marked elevation of TGFβ1 mRNA expression in MAT-LyLu cells (Figure 1.9). While the treatment with 0.25 μM of GP (±) had no significant effect on the expression level of TGFβ1 mRNA. To further study the mechanism by which GP (±) elevates TGFβ1 mRNA, we also measured the total TGFβ1 protein in the conditioned media. The TGFβ1 protein secreted by MAT-LyLu cells were detected in the
conditioned medium (Figure 1.10). TGFβ1 protein is secreted by MAT-LyLu cells in a biologically latent form and that can be activated by transient acidification (Lawrence et al., 1985). The TGFβ Emax™ immunoassay system can measure only biologically active TGFβ1 in our assay. Therefore, all data were generated from acid-activated media. GP (±) treatment significantly increased TGFβ1 secretion in a dose dependent manner. GP (±) at 0.5, 1.0, and 2.0 µM increased TGFβ1 secretion by 1.05 fold, 1.38 fold, and 1.43 fold compared to control respectively. The result suggests that the inhibitory effects of GP (±) on the growth of MAT-LyLu cells are mediated by TGFβ1.

**Effects of TGFβ1 on the proliferation of MAT-LyLu cells**

TGFβ1 exerts its inhibitory effects on cell growth through mechanisms that target proteins such as cyclin D1 and Rb1, which regulate cell cycle progression. The inhibitory effects of GP (±) on MAT-LyLu cell growth are associated with increasing TGFβ1 mRNA expression and TGFβ1 protein secretion, suggesting TGFβ1 as a possible mediator of the inhibitory effects of GP (±) on MAT-LyLu cell growth. To confirm the ability of MAT-LyLu cells to respond to the inhibitory effects of TGFβ1, MAT-LyLu cells were treated with increasing concentrations of TGFβ1 (0, 0.1, 1, and 10 ng/ml) for 24 h. The effect of TGFβ1 on proliferation of MAT-LyLu cells was assessed by thymidine incorporation assay. Results showed that TGFβ1 inhibited the growth of MAT-LyLu cells in a dose-dependent manner (Figure 1.11). The inhibitory effect of TGFβ1 on the MAT-LyLu cells appears to be consistent with our previous report that TGFβ1 is a negative regulator of human prostate cancer cell (PC3) growth (Shidaifat et al., 1996). Based on our previous laboratory results, the ability of MAT-LyLu and PC3
cells to produce TGFβ1 and respond to its inhibitory effects suggests that TGFβ1 is an autocrine regulator of androgen-independent prostate cancer cells. Because of the ability of GP (±) to stimulate TGFβ1 gene expression, it is reasonable to speculate that the inhibitory effects of GP (±) on prostate cancer cell growth may be mediated through augmentation of this inhibitory pathway.

**Effects of GP (±) on the cyclin D1 and Rb1 expression in cultured PC3 cells**

Previously we have shown that GP (±) inhibits the growth of human prostate cancer cells by inducing TGF β1 gene expression (Shidaifat et al., 1996). TGFβ1 is a negative growth regulator that regulates the functions of cyclin D1 and Rb proteins, which are involved in cell cycle progression (Alexandrow et al., 1995; Lalani et al., 1997). Since the antiproliferative effects of GP (±) on human MCF-7 mammary cancer cells is mediated by modulating the expression of Rb and cyclin D1 protein (Ligueros et al., 1997), the mRNA expression of cyclin D1, and Rb1, and cyclin D1 protein expression were determined in GP (±)-treated PC3 cells. The results showed that GP (±) at the concentrations of 1.0 and 2.0 μM resulted in a significant decrease in cyclin D1 mRNA expression and protein expression in PC3 cells in a dose-dependent manner (Figure 1.12 and Figure 1.13). Treatment of PC3 cells with of 0.5 μM GP (±) had no effect on the mRNA and protein expression of cyclin D1. Also, Rb1 mRNA level was not changed by GP (±) treatment in PC3 cells (Figure 1.14). The decrease in cyclin D1 mRNA expression parallels the reduction of DNA synthesis in PC3 cells, suggesting that the inhibitory effects of GP (±) may be mediated by modulating cyclin D1 expression. Modulation of Rb1 mRNA expression does not seem to be involved in the inhibitory
Effects of GP (±), GP (+), and GP (-) on the proliferation of PC3 cells

GP (±) is a racemic mixture of (+) and (-) enantiomers. It has been shown that GP (-) is more potent inhibitor of cell growth than GP (±) in human breast cancer cells (Zhang et al., 1998). To compare the differential effects of GP (±), GP (+), and GP (-) on the proliferation of PC3 cells, the proliferation of PC3 cells after treatment with GP (±), GP (+), and GP (-) on were assessed by thymidine incorporation assay. As shown in Figure 1.15, differential anti-proliferative effects of GP (±), GP (+), and GP (-) at the concentrations of 0.5, and 1.0 μM were observed in PC3 cells. A significant growth inhibition of cells was observed at the 1.0 μM dose of GP (±) and GP (-). However, GP (+) has no significant effects on the proliferation of PC3 cells. Although both GP (-) and GP (±) inhibit the growth of PC3 cells, it seems that GP (-) resulted in a greater inhibition of PC3 cell growth as compared to GP (±), suggesting that GP (-) is more potent than GP (±).

Effects of GP (±), GP (+), and GP (-) on cyclin D1 mRNA expression of PC3 cells

To investigate the mechanism by which GP (±), GP (+), and GP (-) exert differential anti-proliferative actions, the changes in cyclin D1 mRNA expression in PC3 cells in response to treatment with GP (±), and its enantiomers were compared. Figure 1.16 shows that treatment with 1.0 μM of GP (±), and GP (-) resulted in marked reductions of cyclin D1 mRNA expression, while the same concentration of GP (+) had
no effect on cyclin D1 mRNA expression in PC3 cells. These results are consistent with
the anti-proliferative actions of GP (±), and GP (-) shown previously, indicating that the
differential effects of GP (±), and GP (-) may involve the modulation of cyclin D1
mRNA expression in PC3 cells.

Effects of GP (±) on the proliferation of LNCaP cells

LNCaP cell is a human androgen-dependent prostate cancer cell line. To study
the effects of GP (±) on the proliferation of androgen-dependent prostate cancer cells, the
effects of GP (±) on the proliferation of LNCaP cells were assessed by thymidine
incorporation assay. As shown in Figure 1.17, anti-proliferative effects of GP (±) at the
concentrations of 2.0, 4.0, and 8.0 μM were observed in LNCaP cells. GP (±) at 4.0 and
8.0 μM caused significant growth inhibition of LNCaP cells, while GP (±) at the
concentration of 2.0 μM had no significant effects. Our previous results showed that GP
(±) at concentration of 1.0 μM was sufficient to significantly inhibit the growth of PC3
cells. In present study, 4.0 μM of GP (±) was required to achieve a significant level of
growth inhibition of LNCaP cells. This result indicates that LNCaP cells are less
sensitive than PC3 cells to GP (±) treatment.

Effects of GP (±) on cyclin D1 mRNA expression of LNCaP cells

To investigate the mechanism of GP (±)’s anti-proliferative actions in LNCaP
cells, the change in cyclin D1 mRNA expression in LNCaP cells in response to treatment
with GP (±) was measured. RT-PCR results showed that LNCaP had higher cyclin D1
mRNA expression than PC3 cells and that GP (±) at 8.0 μM induced a marked reduction
of cyclin D1 mRNA expression in these cells, while GP (±) at 2.0 and 4.0 μM did not cause significant change (Figure 1.18). This result indicates that the anti-proliferative action of GP (±) on LNCaP cells is partially mediated by modulation of cyclin D1 mRNA expression. Nevertheless, it appears that GP (±) exerts its effects on proliferation of PC3 and LNCaP cells through the same mechanism which favors cell growth inhibition.

Effects of refined cottonseed oil on proliferation of PC3 cells

Cottonseed oil which contains GP (±), a naturally occurring polyphenolic compound has been shown to be a powerful inhibitor of prostate cancer and breast cancer (Shidaifat et al., 1996; Zhang et al., 1998). We investigated the effects of refined cottonseed oil on the proliferation of PC3 cells. The effects of refined cottonseed oil on the proliferation of PC3 cells were assessed by thymidine incorporation assay. As shown in Figure 1.19, anti-proliferative effects of refined cottonseed oil at the concentrations of 5% [0.45 ppm {0.78 μM GP (±)}], and 25% [2.25 ppm {3.86 μM GP (±)}] were observed in PC3 cells. Refined cottonseed oil at the concentration of 1% [0.09 ppm {0.15 μM GP (±)}] had no significant effects on the proliferation of PC3 cells. This finding indicates that cottonseed oil, like GP (±), also possesses anti-proliferative ability.

Effect of refined cottonseed oil on the TGFβ1 mRNA expression and cyclin D1 mRNA expression of cultured PC3 cells

To explore the mechanism of action of cottonseed oil on the proliferation of PC3 cells, we measured the effects of refined cottonseed oil at different concentrations (1%, 5%, and 25%) on TGFβ1 mRNA expression. RT-PCR results showed that refined
cottonseed oil at 25% [2.25 ppm] resulted in a marked elevation of TGFβ1 mRNA expression in PC3 cells (Figure 1.20), while the treatment of PC3 cells with refined cottonseed oil at 1% (0.09 ppm) and 5% (0.45 ppm) had no effects on the expression level of TGFβ1 mRNA. This result is consistent with the anti-proliferative effects of refined cottonseed oil, and indicates that the anti-proliferative ability of refined cottonseed oil in PC3 cells is associated with enhanced TGFβ1 mRNA expression. To further study the mechanism of action of refined cottonseed oil, we also measured the mRNA expression of cell cycle regulator, cyclin D1, in PC3 cells treated with refined cottonseed oil. The results showed that refined cottonseed oil at the concentration of 25% (2.25 ppm) resulted in a significant decrease in cyclin D1 mRNA expression in PC3 cells (Figure 1.21), while refined cottonseed oil at 1% (0.09 ppm) and 5% (0.45 ppm) had no effect on the mRNA expression of cyclin D1 in PC3 cells. The decrease in cyclin D1 expression parallels the reduction of DNA synthesis in PC3 cells, suggesting that the inhibitory effects of refined cottonseed oil on PC3 cells may be related to stimulation of TGFβ1, which in turn modulates the expression of cyclin D1. Cottonseed oil, like GP (±), exerts its anti-proliferative effects through the TGFβ1 pathway. These results indicate that food-grade GP (±)-containing cottonseed oil also possesses potent anti-proliferative abilities against prostate cancer cells in vitro.

**Discussion**

GP (±) is a yellowish polyphenolic pigment that occurs naturally in cottonseed and is also found in cotton plant by-products (Jaroszewski et al., 1990) 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 laboratory and other laboratories have shown that GP (±) can inhibit the growth of human prostate cancer cells (Shidaifat et al., 1996), human BPH cells (Shidaifat et al., 1997), human ovarian cancer cells (Wang and Rao., 1984), colon cancer cells (Tuszynski and Cossu., 1984; Wang et al., 2000) and human breast cancer cells (Hu et al., 1994). Many studies have shown that GP (-) possesses more potent anti-proliferative ability than its parent compound when it was tested in human skin fibroblasts (Joseph et al., 1986), melanoma cell lines (Blackstaffe et al., 1997), ovarian cancer cell lines (Band et al., 1989 and breast cancer cells (Liu et al., 2002). 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 (Liu et al., 2002). In the present study, GP (-) and GP (±) at a concentration of 1.0 μM of significantly inhibited the growth of PC3 cells. Although both GP (-) and GP (±) inhibited the growth of PC3 cells, GP (-) resulted in greater inhibition as compared to GP (±). Consistent with its ability to inhibit PC3 cells proliferation, our results indicated that GP (±) inhibited the DNA synthesis of both PC3 cells and MAT-LyLu cells, and prolonged DTs of both cell types in a dose-dependent manner. In addition, GP (±) at 4.0 μM significantly inhibited the DNA synthesis of LNCaP cells, suggesting that GP (±) may inhibit both human androgen-dependent and androgen-independent prostate cancer cell growth. Nevertheless, it seems that LNCaP cells are less sensitive to the inhibitory effect of GP (±). In contrast, cultured MAT-LyLu cells were 4 fold and 16 fold more sensitive to GP (±) treatment with respect to proliferation than PC3 and LNCaP cells.
respectively. GP (±) at 0.25 μM was sufficient to achieve a significant growth inhibition of MAT-LyLu cells, while PC3 and LNCaP cells required treatment with 1.0 μM and 4.0 μM concentrations to achieve similar levels of inhibition, respectively. In the androgen-independent prostate cancer cells, it seems that the faster the cells grow, the more sensitive the cells are to GP (±) treatment. For example, the proliferation rate of MAT-LyLu cells was about 8.1-fold higher than that of PC3 cells (9.4x10^3 DPM/well vs 11.6x10^3 DPM/well in DNA synthesis). Similarly the DT of MAT-LyLu cells is shorter than that of PC3 cells (8.9 h vs 22.48 h). Interestingly, both GP (-) enantiomers and cottonseed oil possessed anti-proliferative effects on PC3 cells. PC3 cell proliferation was significantly suppressed by treatment with GP (-) at 1.0 μM, and refined cottonseed oil at 5%. Both GP (-) and refined cottonseed oil exhibited a greater potency than GP (±).

In the U.S., cottonseed oil and GP (±) directly enters the human diet through its use in cooking, frying, and food processing, and its presence in salad dressing, shortening, margarine, canned foods and snack foods. Indeed, cottonseed oil has been the golden standard for the snack food industry due to its desired oxidative stability and flavor quality. However, since GP (±) is considered an undesirable ingredient in cottonseed products, the FDA (1974) limits the free GP (±) content in cottonseed products that are destined for human and animal consumption to no more than 450 ppm (0.045%) (Food and drug Administration., 1974). As a result, the United States Department of Agriculture (U.S.D.A.) and the cotton industry spend millions of dollars to improve the techniques of removing GP (±) from cottonseed products to meet FDA regulations. In 1999, an estimated 44,000 metric tons of free GP (±) was removed as
waste during the processing of cottonseed products. The production of cottonseed in 1999 was estimated at 6.4 million metric tons with an economic value of $4.5 billion (Calhoun et al., 2000). GP (±)-containing milk (GP-milk), which was collected from Brown Swiss dairy cows treated daily with federally allowable 450 ppm of GP (±) for 6 days, significantly inhibits the rate of \(^3\)H-thymidine incorporation in MCF-7, MCF-7 Adr, and RE-B2T cell lines in a dose-dependent manner (Hu et al., 1994). We have also shown that PC3 cell proliferation is significantly suppressed by treatment with 5% refined cottonseed oil (Jiang et al., 2000). From our extensive experience in GP (±) research, the effective antiproliferative dose range of pure GP (±) is 1-5 \(\mu\)M. Thus, the cottonseed oil exhibited an inhibitory dose that represents a greater potency than that of purified GP (±) (GP acetic acid). The fact that refined cottonseed oil possesses extremely potent anti-proliferative activity against prostate cancer cells in vitro indicates that cottonseed oil may serve as a dietary chemopreventive agent against prostate cancer.

Although our previous research has shown that GP (±) inhibited 5α reductase activity and 3α-hydroxysteroid dehydrogenase activity in rat testes (Moh et al., 1993), and induced spermidine/spermine N\(^1\)-acyetyltransferase in canine prostate epithelial cells (Chang et al., 1997), the mechanism of the inhibitory effect of GP (±) is not clear. Our previous reports showed that GP (±) could inhibit cell growth by inducing TGFβ1 mRNA and blocking the cell cycle at the \(G_0/G_1\) phase in human prostate cancer and human BPH cells (Shidaifat et al., 1996; Shidaifat et al., 1997). Our previous results also showed that the anti-proliferative effect of GP (±) might be mediated by inducing TGFβ1 protein production in the stromal cells isolated from human breast adipose tissues (Zhang et al., 1998). Present results showed that TGFβ1 mRNA expression was present in PC3 cells
and MAT-LyLu cells, but absent in LNCaP cells (data not shown). GP (±) treatment markedly elevated TGFβ1 mRNA expression of both PC3 cells and MAT-LyLu cells, while refined cottonseed oil also increased the TGFβ1 mRNA expression of PC3 cells. ELISA results showed that GP (±) stimulated TGFβ1 secretion of PC3 cells as well as MAT-LyLu cells after 24-hour incubation. We also found that TGFβ1 significantly inhibited the growth of MAT-LyLu and PC3 cells in a dose-dependent manner. In addition, we also investigated other important cell cycle regulators (cyclin D1 and Rb1) for their role in mediating the effects of GP (±) on the growth of prostate cancer cells. It is known that cyclin D1 and Rb protein are involved in mediating the action of GP (±) in the MCF-7 human breast cancer cell line (Ligueros et al., 1997). Our results showed that GP (±), GP (-) and refined cottonseed oil treatments decreased cyclin D1 mRNA expression in PC3 cells, while GP (±) caused the reduction of cyclin D1 mRNA expression in LNCaP cells at a higher dose (8.0 μM). In contrast, GP (±) had no effect on the Rb1 mRNA expression in PC3 cells. These findings suggest that the anti-proliferative effects of GP (±) and refined cottonseed oil be mediated by inducing TGF β1 gene expression, which further regulates the involvement of cyclin D1 protein in cell cycle progression. Western blot results further confirmed our hypothesis about that GP (±) exerts its effects through TGFβ1 regulating the function of cyclin D1 protein. GP (±) probably exerts its effect at the transcriptional level, either by increasing transcription or by modifying the stability of TGFβ1 mRNA within the cell cycle regulatory pathway (Ravitz et al., 1997). 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 and MAT-LyLu cells. As TGFβ1 affects cell cycle-regulating proteins, such as cyclin D1 and
Rb proteins, which are involved in cell cycle progression from G1 phase to S phase, this finding further suggests that TGF β1 is a potential physiological regulator of normal prostate cells, cancer cells and human breast cancer cells. In addition, it is known that TGF β1 prevents phosphorylation of Rb protein which would increase c-myc expression and accelerate proliferation (Alexandrow et al., 1995; Lalani et al., 1997). A report demonstrated that GP (+) induced cell cycle arrest at G1/S phase by decreasing Rb protein expression, Rb protein phosphorylation, and cyclin D1 protein expression in MCF-7 cells (Ligueros et al., 1997). It has also been reported that TGFβ1 treatment reduced cyclin D1 mRNA and protein expression in rat intestinal epithelial cells (Ko et al., 1995) and expression of Rbl and Rb protein phosphorylation in Mv1Lu cell (Schwarz et al., 1995). These findings led us to test whether GP (+) could affect the mRNA and protein expression of cyclin D1 and Rbl mRNA expression in PC3 cells. The results of our experiment demonstrated that GP (+) could reduce the cyclin D1 mRNA expression and protein expression of PC3 cells, but has no influence on Rbl mRNA expression. This finding suggests that GP (+) induced TGFβ1 secretion which reduced cell cycle protein, cyclin D1, and further modulated cell cycle progression in PC3 cells. These findings suggest that GP (-) and GP (+) may become chemopreventive and chemotherapeutic agents against human prostate cancer.

TGFβ1 is an important growth inhibitor of a variety of cancer cells (Knabbe et al., 1987; Lalani et al., 1997). Under our experimental conditions, our results showed that TGFβ1 is able to significantly inhibit the growth of human prostate cancer PC3 cells in a dose-dependent manner. TGFβ1 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 TGFβ1 suggest that TGFβ1 can function as a negative autocrine growth regulator for PC3 cells. We have demonstrated that the addition of 15% conditioned media (containing 105.4 pg/ml of TGFβ1) significantly inhibited the proliferation of PC3 cells by 18.9% compared with the control group. When anti-TGFβ1-β2-β3 antibody at 25 µg/ml was added to the conditioned media, the growth inhibition of PC3 cells induced by 15%-conditioned media (105.4 pg/ml of TGFβ1) was completely reversed. These results along with the observations that GP (±) significantly increased TGFβ1 secretion and TGFβ1 gene expression strongly support the hypothesis that the anti-proliferative activity of GP (±) is mediated by TGFβ1 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 (Tso et al., 1984), and MAT-LyLu cells transplanted in Copenhagen rat, and prolonged the survival of mice implanted with mouse mammary carcinoma 755 cells (Rao et al., 1985). GP (±) caused a reduction in the lung and lymph node metastasis of MAT-LyLu-bearing Copenhagen rats and caused a decrease in the invasive ability of MAT-LyLu cells in vitro (Chang et al., 1993; Jiang et al., 2000). GP (±) caused the complete resolution of pulmonary metastases and >50% reduction in the size of hepatic metastases in a human adrenocortical carcinoma patient (Flack et al., 1993). The susceptibility of the multidrug-resistant human breast cancer cell line, MCF-7 Adr, to the cytotoxic effects of GP (±) was found to be due mainly to GP (±)'s ability to inactivate the P-170 drug efflux pump and its lipophilicity in the lipid membrane (Jaroszewski et al., 1990). The P-170 is an energy-dependent cell membrane glycoprotein that is theorized to be responsible for maintaining drug concentrations in resistant cells at sublethal levels. GP (±) is a lipophilic compound
and its effects on cancer cells may be due to its lipophilicity and consequent effects at the level of the cell membrane. The strong interaction of GP (±) with lipid bilayers has been noted for years (Reyes et al., 1984; 1986; De Peyster et al., 1986). Whether GP (±) is capable of exerting a similar anti-multidrug resistance effect in human prostate cancer cells remains to be investigated.

In a chronic oral trial in man, GP (±) did not result in myelosuppression (Qian et al., 1984). Furthermore, GP (±) caused the tumor regression in advanced cancer patient with gliomas (Bushunow et al., 1995), adrenal cell carcinoma (Flack et al., 1993), and breast cancer (Poznak et al., 2001) that was refractory to standard therapy. GP (-), an enantiomer of racemic GP (±), was more potent than cisplatin, melphalan and dacarbazine in the melanoma lines, cisplatin and dacarbazine in the lung cancer lines (Shelley et al., 1990). Our results also confirmed the anti-proliferative activity of GP (-) and cottonseed oil in PC3 cells and found that the anti-proliferative activities of GP (-) and cottonseed oil may be associated with the reduction of cyclin D1 gene expression. These results have indicated that GP (-) and refined cottonseed oil have a potential value as chemopreventive agents against prostate cancer.

TGFβ exerts its reversible cell cycle arrest largely through maintaining E2F bound to Rb in a transcriptionally silent form. TGFβ also inhibits the cyclin-Cdk complex through regulating the expression of cyclin-Cdk inhibitor (CKIs). p15^{ink4B} and p21^{cip1} are cyclin-Cdk inhibitors. p15^{ink4B} can inhibit the phosphorylation of the complexes of cyclinD-Cdk4 and cyclinD-Cdk6, while p21^{cip1} can inhibit Cdk2, Cdk4, and Cdk6 and is capable of inducing cell cycle arrest in G1 phase. TGFβ can induce p15^{ink4B} and p21^{cip1} expression to arrest cell cycle in G1 phase. Thus, the known and/or
the potential sites of GP’s action are following: Rb, cyclin D1, p15\(^{ink4B}\) and p21\(^{cip1}\), and c-Myc. Because our laboratory has already shown that GP (±) can induce the production of TGFβ1 in PC3 cells (Shidaifat et. al., 1996). In addition, other researchers have reported that GP (±) at 5\(\mu\)M inhibits the expression of cyclin D1 and increases phosphorylated Rb (Ligueros et. al., 1997). Thus, the inhibitory effect of GP (±) on prostate cancer cells may be mediated by multiple mechanisms such as inhibition of the expression of cyclin D1 and c-Myc, and the induction of p15\(^{ink4B}\) and p21\(^{cip1}\). Our future research will be directed at the study of the effects of GP (±) and its analogues on these cell cycle regulators so that we can further understand the mechanism of the action of these compounds on prostate cancer cells. Such research enable us to develop a new agent to specifically target a cell cycle regulator in prostate cancer cells. An agent that specifically targets cell cycle regulator could be clinically useful in the treatment of prostate cancer. Thus, the effects of GP (±) on the c-Myc, p15\(^{ink4B}\) and p21\(^{cip1}\) need to be elucidated.

Although androgen ablation is a primary method for prostate cancer treatment, patient’s response is temporary. This transient response to androgen withdrawal is due to transition from androgen-dependent cancer cells to androgen-independent cancer cells (Isaacs et al., 1981; Isaacs et al., 1993). 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 (Isaacs et al., 1993). Our results indicated that GP (±) and GP (-) are potent inhibitors of androgen–independent prostate cancer cells. In addition, the ability of GP (±) to inhibit 5-α reductase within the testes (Moh et al., 1993) suggests the effectiveness against
androgen–dependent prostate cancers. Thus, GP (±), GP (-), and cottonseed oil could be potent chemopreventive agents against androgen-dependent and -independent prostate cancer cells.

Agents that would increase tumor sensitivity to antineoplastic therapy and decrease drug resistance are very important. The enzyme aldehyde dehydrogenase class 1 and class 3 catalyze the detoxification of cyclophosphamide and other oxazaphosphorines (Poznak et al., 2001). Any agent that inhibits aldehyde dehydrogenase class 1 and class 3 will increase the efficacy of cyclophosphamide and other oxazaphosphorines. GP (±) is such a compound that has been shown to inhibit aldehyde dehydrogenase class 3 in cultured human breast adenocarcinoma, thereby increasing the sensitivity of these cultured cells to oxazaphosphorines (Sreerama et al., 1997; Rekha et al., 1996). This suggests that GP (±) may augment the therapeutic efficacy of oxazaphosphorine agents. From a published clinical study, GP (±) is safe and well tolerated in metastatic breast cancer patients when used alone (Poznak et al., 2001). Therefore, the effects of GP (±) in combination with other anticancer agents may be an important application for the treatment of prostate cancer patients. Furthermore, our results showed that the inhibitory effects of GP (-) on the growth inhibition of prostate cancer cells are more potent than that of GP (±). This means that a new generation of GP (-)-like compounds should have higher specificity and sensitivity, and/or less toxicity in prostate cancer cells, resulting in few side effects. Thus, the modifications of the structure of the GP (-) enantiomer may help us to understand the relationship between structure and function and produce highly specific GP (-)-like compounds against human prostate cancer. Meanwhile, tailor-made diets or snacks can be developed for chemopreventive purpose in which the critical
ingredient is cottonseed oil or cottonseed meal.
Figure 1.1. Effects of GP (±) on the proliferation of PC3 cells. Effects of GP (±) on the proliferation of PC3 cells as 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 * represent 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 1.1

![Bar chart showing thymidine incorporation (CPM/well) at different gossypol concentrations (µM). The chart indicates a significant decrease in thymidine incorporation with increasing gossypol concentration. The data is represented as Mean±SD, with an asterisk (*) indicating a P-value <0.05. N=4 for each concentration.](image-url)
Figure 1.2 Effects of GP (±) on the doubling time of PC3 cells. Effects of GP (±) treatment on the cell growth of PC3 cells as 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 times after treatment (0, 12, 24, 36, 48, 60, and 72 h). The trypan blue dye-exclusion method was used to evaluate the cell viability. The cells were examined in a counting chamber with a light microscope. Only viable cells were recorded. Each bar represents the mean ±SD of 4 wells. Bar with * represent 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 PC3 cells doubling time was calculated using Chopra’s method (Chopra et al., 1996). The DTs during the exponential growth phase were determined as $N=N_02^n$ where $N_0$ is the initial population and $N$ is the final population after “n” doublings. The time (g) for the population to double 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.
Figure 1.2

Dosage Concentration (µM)

0.0 0.5 1.0 2.0

Doubling Time (hours)

* * *
Figure 1.3 Effects of GP (±) on the TGFβ1 mRNA expression of PC3 cells. The TGFβ1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with GP (±) at 0.0, 0.5, 1.0, 2.0 μM for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of TGFβ1 to β-actin. Each bar represents the Mean±SD of 3 replicate samples. Bar with * represent 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 1.3
Figure 1.4. Effects of GP (±) on TGFβ1 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 this treatment period, TGFβ1 secretion was measured by TGFβ1 Emax™ ImmunoAssay System using acid-activated conditioned media from PC3 cells with the GP (±) treatment. 1x10⁴ PC3 cells contains about 30 μg protein. Each bar represents the mean ±SD of 4 wells. Bar with * represent 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 1.4

Mean±SD
N=4 (well)
*P<0.05
Figure 1.5 Effects of TGFβ1 on the proliferation of PC3 cells. Effects of TGFβ1 on proliferation of PC3 cells as assessed by thymidine incorporation assay. PC3 cells were treated with 0.0, 0.1, 1.0, and 10 ng/ml of TGFβ1 for 24 h. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 1.5

Thymidine Incorporation (1×10^3 DPM/well)

TGFB1 concentration (ng/ml)

N=4 (well)
Mean±SD
*P<0.05
Effects of conditioned media harvested from gossypol treated PC3 cells on the proliferation of PC3 cells.

Effects of 24-hour serum-free conditioned media collected from cultured PC3 cells on the proliferation of PC3 cell line, a human prostatic cancer cell line originally derived from prostatic epithelial cells, were tested. The detail of preparation of conditioned media from PC3 cells were described in Materials and Methods section of this chapter. PC3 cells were seeded into 24-well plates. Each treatment group with diluted conditioned media (1 to 15%) in RPMI-1640 medium containing 5% FCS were cultured with or without 25 μg/ml anti-TGF-β1, -β2, -β3 antibody for 24 h. The proliferation of PC3 cells was determined by measuring the incorporation of thymidine. Thymidine uptake levels were expressed as the percentage of thymidine incorporated in treatment group over the control group. The result showed that 25 μg/ml anti- TGF-β1, -β2, -β3 antibody was sufficient to block the action of TGFβ1 produced by conditioned media-treated PC3 cells. Each bar represents the mean ± SD of 3 experiments. Bar with * represent 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 1.6

TGFB1-ß2-ß3 antibody concentration=25 μg/ml
TGFB contained in conditioned medium (pg/ml)
1%: 7.03 pg/ml
5%: 35.13 pg/ml
15%: 105.4 pg/ml

N=4 (well)
Mean±SD
*P<0.05
Figure 1.7. Effects of GP (±) on the proliferation of MAT-LyLu cells. Effects of GP (±) on the proliferation of MAT-LyLu cells as assessed by thymidine incorporation assay. MAT-LyLu cells were treated with 0.0, 0.25, 0.5, and 1.0 µM of GP (±) for 24 h. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 1.7

Thymidine incorporation (DPM/well)

Crosspool concentration (µM)

0.0

0.05

0.25

1.0

N = 4 (well)

Mean ± SD

P < 0.05

DF: 3
Figure 1.8. Effects of GP (±) on the doubling time of MAT-LyLu cells. Effects of GP (±) treatment on the cell growth of MAT-LyLu cells as assessed by doubling time assay. MAT-LyLu cells were treated with different concentrations of GP (±) (0.0, 0.25, 0.5, and 1.0 μM). Cell numbers were determined using a hemacytometer and the trypan blue dye-exclusion method at different times after treatment (0, 12, 24, 36, 48, 60, and 72 h). Each bar represents the mean ±SD of 4 wells. Bar with * represent 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 MAT-LyLu cells doubling time was calculated using Chopra’s method (Chopra et al., 1996). The DTs during the exponential growth phase were determined as $N=No \times 2^n$ where $No$ is the initial population and $N$ is the final population after “n” doublings. The time (g) for the population to double 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 $No$ is determined.
Figure 1.8

![Bar graph showing the effect of different concentrations of Gossypol on the doubling time of a cell population. The graph displays the mean ± standard deviation (Mean±SD) for N=4 replicates. Statistical significance is indicated by an asterisk (*) where *P<0.05.](image-url)
Figure 1.9. Effects of GP (±) on the TGFβ1 mRNA expression of MAT-LyLu cells.
The TGFβ1 mRNA expression of MAT-LyLu cells was measured by RT-PCR analysis. MAT-LyLu cells were treated with 0, 0.25, 0.5, 1.0 μM of GP (±) for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of TGFβ1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent 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 1.9

TGF-β1 (782 bp)  
β-actin (593 bp)

N = 3  
Mean ± SD  
*P < 0.05

Relative expression ratio (TGFβ1/β-actin)

0.0  0.5  1.0  2.0
Gossypol concentration (μM)
Figure 1.10. Effects of GP (±) on TGFβ1 secretion in MAT-LyLu cells. MAT-LyLu cells were treated with different concentrations of GP (±) (0.0, 0.5, 1.0, and 2.0 μM) for 24 h. At the end of this treatment period, TGFβ1 secretion was measured by TGFβ1 Emax™ ImmunoAssay System using acid-activated conditioned media from MAT-LyLu cells with the GP (±) treatment. 1x10^5 MAT-LyLu cells contain about 20 μg protein. Each bar represents the mean ±SD of 4 wells. Bar with * represent 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 1.10
Figure 1.11. Effects of TGFβ1 on the proliferation of MAT-LyLu cells. Effects of TGFβ1 on proliferation of MAT-LyLu cells as assessed by thymidine incorporation assay. MAT-LyLu cells were treated with 0.0, 0.1, 1.0, and 10 ng/ml of TGFβ1 for 24 h. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 1.11
**Figure 1.12. Effects of GP (±) on the cyclin D1 mRNA expression of PC3 cells.** The cyclin D1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with GP (±) at 0.0, 0.5, 1.0, 2.0 μM for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of cyclin D1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent 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 1.12
Figure 1.13. Effects of GP (±) on the cyclin D1 protein expression of PC3 cells.

Effects of GP (±) on the cyclin D1 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 D1 protein expression. Western blot analysis was performed as described in Materials and Methods section in this chapter. Total proteins were separated on ready gel Tris-HCl gel and transferred onto a PVDF (polyvinylidene difluoride) membrane. Cyclin D1 protein was detected using mouse anti-human cyclin D1 gene product monoclonal IgG. In PC3 cells, an approximately 36 kDa protein was detected. The size of this protein corresponds to that of cyclin D1.
Figure 1.13
Figure 1.14. Effects of GP (±) on the Rbl mRNA expression of PC3 cells. The Rbl mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with GP (±) at 0.0, 0.5, 1.0, 2.0 μM for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of Rbl to β-actin. Each bar represents the Mean ± SD of 3 replicate samples.
Figure 1.14

Relative expression ratio
(Rb1/β-actin)

Gossypol concentration (µM)

N=3
Means±SD

β-actin (593 bp)
Rb1 (259 bp)
Figure 1.15. Effects of GP (±), GP (+), and GP (-) on the proliferation of PC3 cells.

Effects of GP (±), GP (+), GP (-) on the DNA synthesis of PC3 cells as assessed by thymidine incorporation assay. PC3 cells were treated with GP (±), GP (+) and GP (-) at 0.0, 0.5, and 1.0 µM for 24 h respectively. Each bar represents the Mean ± SD of 4 wells. Bar with * represent 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 1.15
Figure 1.16. Effects of GP (±), GP (+), GP (-) on the cyclin D1 mRNA expression of PC3 cells. The cyclin D1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with GP (±), GP (+), GP (-) at 0.0, 0.5, and 1.0 μM for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of cyclin D1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent 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 1.16
Figure 1.17. Effects of GP (±) on the proliferation of LNCaP cells. Effects of GP treatment on the proliferation of LNCaP cells as assessed by thymidine incorporation assay. LNCaP cells were treated with 0.0, 2.0, 4.0, 8.0 μM of GP (±) for 24 h. Each bar represents the Mean ± SD of 4 wells. Bar with * represent 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 1.17

- N=4 (well)
- Mean±SD
- *P<0.05
Figure 1.18. Effects of GP (±) on the cyclin D1 mRNA expression of LNCaP cells.

The cyclin D1 mRNA expression of LNCaP cells was measured by RT-PCR analysis. LNCaP cells were treated with 0.0, 2.0, 4.0, 8.0 μM of GP (±) for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of cyclin D1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent 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 1.19. Effects of refined cottonseed oil on the proliferation of PC3 cells.

Effects of refined cottonseed oil on the proliferation of PC3 cells as assessed by thymidine incorporation assay. PC3 cells were treated with 1%, 5% and 25% of refined cottonseed oil or with 1%, 5%, 25% of corn oil as a control for 24 h. Each bar represents the Mean ± SD of 4 wells. Bar with * represent 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 1.19
Figure 1.20. Effects of refined cottonseed oil on the TGFβ1 mRNA expression of PC3 cells. The TGFβ1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with 1%, 5%, 25% of refined cottonseed oil or 1%, 5% and 25% of corn oil for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of TGFβ1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent means that are significantly different from the control group. A probability (p) of less than 0.05 was considered statistically significant (P<0.05).
N=3
Means±SD
*P<0.05

Figure 1. 20 Oil concentration (%)

Relative expression ratio (TGFβ1/β-actin)
Figure 1.21. Effects of refined cottonseed oil on the cyclin D1 mRNA expression of PC3 cells. The cyclin D1 mRNA expression of PC3 cells was measured by RT-PCR analysis. PC3 cells were treated with 1%, 5%, 25% of refined cottonseed oil or 1%, 5% and 25% of corn oil for 24 h. RT-PCR was performed as described in Materials and Methods section in this chapter. β-actin was used as an internal loading control. Quantification of RT-PCR product was based on the density of the specific PCR bands in ethidium-bromide stained agarose gel. The results are expressed as the relative expression ratios of cyclin D1 to β-actin. Each bar represents the Mean ± SD of 3 replicate samples. Bar with * represent 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 1.21

- β-actin (593 bp)
- Cyclin D1 (372 bp)
- β-actin (593 bp)
- Cyclin D1 (372 bp)

Mean ± SD
P < 0.05

Oil concentration (%)

- Corn oil
- Refined cotton seed oil

N = 3
Mean ± SD
*P < 0.05

Relative expression ratio (Cyclin D1/β-actin)

1 5 25

0.0 0.3 0.6 0.9
CHAPTER 2

THE EFFECTS OF GOSSYPOL ON THE INVASIVENESS OF MAT-LyLu CELLS and MAT-LyLu CELLS FROM THE METASTASIZED LUNGS OF MAT-LyLu-BEARING COPENHAGEN RATS

Abstract

We isolated a novel subline of the MAT-LyLu cell line from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats (MLL cells). In this study, we compared the MLL cells to the parental MAT-LyLu cells with respect to invasive ability, mRNA expression level for the nm23 metastasis suppressor gene, and response to racemic gossypol [GP (±)], a natural compound with documented antiproliferative and antimetastatic activity, in an in vitro invasion assay. MLL cells were isolated from mechanically dissociated metastasized lungs from MAT-LyLu-bearing Copenhagen rats. Comparisons of invasive ability and steady-state levels of nm23 mRNA between MLL and MAT-LyLu cells were determined by in vitro invasion assay and RT-PCR, respectively. The results showed that MLL cells displayed a higher penetration percentage than MAT-LyLu cells in the in vitro invasion assay. Furthermore, RT-PCR
revealed that MLL cells possessed lower steady state levels of nm23 mRNA than MAT-LyLu cells, suggesting a molecular basis for the observed differences in the in vitro invasive ability. Finally, both MLL and MAT-LyLu cells were susceptible to GP (±), which induced dose-dependent inhibition of invasive activity with the elevation of nm23 metastasis suppressor gene expression. This invasive inhibition of GP (±) may be mediated by nm23 metastasis suppressor gene. These results report the isolation of a novel, more highly invasive subline of the MAT-LyLu cell line that is as susceptible to the inhibitory effects of GP (±) as the parental MAT-LyLu cells. The MLL cells, in combination with the parental MAT-LyLu cells, can be valuable tools for investigating the biology and behavior of metastatic cells and their response to chemotherapeutic/preventive agents.

**Introduction**

Prostate cancer is the leading cause of cancer-related mortality in American men over the age of 45 years due to its unpredictable hormonal independence and highly metastatic nature (Paganini-Hill et al., 1988). The metastasis of prostate cancer is a major cause of mortality. The major metastatic sites of prostate cancer are lung, lymph nodes, and bone marrow (Bostwick et al., 1993). An understanding of the metastatic process in prostate cancer is very important. The nm23 gene was originally isolated from a murine melanoma cell line (Steeg et al., 1988). The down-regulation of nm23 expression correlates with metastatic potential in some tumors and cell lines (Steeg et al., 1988; Bevilacqua et al., 1989; Yamaguchi et al., 1993). The nm23 gene is a metastasis
suppressor gene and the transfection of nm23 cDNA suppresses metastasis potential of human prostate cancer cells (Lim et al., 1998). While the nm23 gene suppresses tumor metastasis, it does not alter the growth rate and tumorigenicity of tumor cells (Rinker-Schaeffer). It was found that the transfected nm23 gene inhibits the ability of prostate cancer cells to adhere to extracellular matrix and consequently reduces metastasis of human prostate cancer (Lim et al., 1998). Therefore, the nm23 gene has been used as a measure of metastatic potential (Ichikawa et al., 1992; Lee et al., 1999; Wang et al., 1997).

Racemic gossypol [GP (±)], a polyphenolic compound naturally occurring in cottonseed, originally served as a potent antifertility agent in males and females (Lin et al., 1987a; Gu et al., 1990; Gu et al., 1991) and subsequently has gained interest as an anticancer agent in a variety of human cancer cell lines including those of the breast, ovary, cervix, uterus, adrenals, pancreas and colon (Band et al., 1989; Benz et al., 1987; Benz et al., 1988; Coyle et al., 1994; Jaroszewski et al., 1990; Liang et al., 1995; Thomas et al., 1991; Tuszynski et al., 1984; Wang et al., 1984; Wu et al., 1989). We have shown that GP (±) can inhibit the in vitro growth of PC3, MCF-7 cells and primary cultured human prostate cells, as well as the in vivo growth and metastasis of MAT-LyLu cells after implantation into Copenhagen rats (Shidaifat et al., 1996; Shidaifat et al., 1997; Zhang et al., 1998; Chang et al., 1993). GP (±) has exhibited efficacy against the multidrug-resistant human breast cancer cell line, MCF-7 Adr. GP (±)’s cytotoxic effects on this cell line were found to be due primarily to its ability to incapacitate the P-170 drug efflux pump, an energy–dependent cell membrane glycoprotein thought to maintain sublethal drug concentrations in resistant cells, as well as its lipophilicity and consequent
effects at the level of the cell membrane (Jaroszewski et al., 1990). Indeed, the strong interaction of GP (±) with lipid bilayers has been noted for years (Reyes et al., 1984; Reyes et al., 1986; De Peyster et al., 1986a). This inhibitory effect of GP (±) on the drug-resistant MCF-7 Adr cells may prove to be important for the development of new therapeutic strategies for cancer patients who are refractory to conventional therapies. In addition, using the chicken embryo chorioallantoic membrane assay for angiogenic activity, we determined that exposure to a GP (±)-impregnated nitrocellulose membrane (200 μg, 48h) reduced microvessel formation (Hu et al., 1995). Overall, our findings suggest that GP (±) can reduce metastasis and angiogenesis which are two of the most critical clinical problems facing prostate and breast cancer patients and their clinicians.

The mechanism(s) of GP (±)’s antiproliferative effects on cancer cells is still unknown, but they seem to be related to cell cycle arrest (Shidaifat et al., 1997; Zhang et al., 1998). We have shown that GP (±) induces wild type p53 protein secretion in MCF-7 cell (Sugimoto et al., 1997), and that GP (±) induces arrest of PC3 cells (Shidaifat et al., 1996) and primary cultured human BPH cells (Shidaifat et al., 1997) at the G0/G1 phase of the cell cycle. This GP (±)-induced cell cycle arrest was associated with enhanced TGFβ gene expression and TGFβ protein production (Shidaifat et al., 1997; Zhang et al., 1998). Consistent with our findings is a report demonstrating that GP (±) suppresses MCF-7 cell growth by modulating the expression of the cell cycle regulatory proteins, Rb and cyclin D1 (Ligueros et al., 1997).

The MAT-LyLu cell line was derived originally from the Dunning R3327H prostate cancer cell line. After 20 years of in vivo selection, MAT-LyLu cells are fast growing, androgen-independent, highly metastatic, and simulate stages II-IV of human
prostate cancer. Because of its rapid metastatic capacity to regional lymph nodes and lung, the MAT-LyLu cell line provides one of the few models to study the metastatic process of prostate cancer (Shaw et al., 1986; Shaw et al., 1987a; Shaw et al., 1987b).

Recently, we isolated a novel subline of the MAT-LyLu cell line from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats (MLL cells). To characterize the behavior of this novel subline, as well as the inhibitory effects of GP (±) on the invasiveness of both MAT-LyLu and MLL cells, the present study compared the two cell lines with respect to invasive ability, mRNA expression level for the nm23 metastasis suppressor gene, and response to GP (±) in an in vitro invasion assay.

**Materials and Methods**

*Reagent.*

Racemic gossypol (Sigma Chemical Co., St. Louis, MO) was dissolved in 100% ethanol to make a 10 mM stock solution. Treatment solutions were prepared by the dilution of stock solution in RPMI-1640 medium with 5% FCS.

*Cell culture, implantation, and treatment.*

MAT-LyLu cells were cultured as previously described in Chapter 1. The procedure of isolating a novel subline of the MAT-LyLu cell line from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats (MLL cells) was summarized in Figure 2.1. Each Copenhagen rat received a subcutaneous injection of 0.75 x 10^6 viable MAT-
MAT-LyLu prostate cancer cells

15 days

Primary tumor mass

Metastasized lymph nodes

Metastasized lung

Isolation of cancer cells from metastasized lung

Culture

15 days

Pure cancer cells isolated from metastasized lung

Cultured transplantable cancer cells in rats

In vitro invasion experiments

Metastatic Studies

nm23 gene expression experiments

Figure 2.1. Procedures of isolation of MLL cells
LyLu cells in the flank region. Fifteen days after implantation, animals were sacrificed and the tumor tissues (about 30 g) and lung tissues were collected for in vitro studies. The lung tissues were minced, without enzymatic digestion, and cultured in RPMI-1640 supplemented with 5% FCS. After six to eight generations in culture under these conditions, the surviving cancer cells isolated from the lungs of MAT-LyLu-bearing Copenhagen rats were termed MLL cells. Confirmation that MLL cells were metastasized cancer cells originating from the parental MAT-LyLu cell line was achieved by the subcutaneous implantation of two groups of Copenhagen rats with 0.75 x 10^6 viable MLL cells or identically prepared lung cells from untreated Copenhagen rats. Fifteen days after implantation, the tumors were present in the rats injected with the MLL cells, while no tumors were observed in animals that received the normal lung cells. MLL cells were cultured in RPMI-1640 (GibcoBRL) containing an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin) (GibcoBRL) and 5% fetal calf serum (FCS) in a humidified incubator (37°C, 5% CO₂ 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). The dissociated cells were pelleted by centrifugation at 200xg for 5 min and then resuspended in RPMI-1640 supplemented with 5% FCS.

*In vitro invasion assay.*

In vitro invasion assays were carried out by methods based on those described previously (Albini et al., 1987) in the 24-well Transwell™ system (pyrocarbonate filter, 8
μm pore size; Costar Co., Cambridge, MA). Cell culture inserts (6.5-mm-diameter) were converted into invasion chambers by applying a layer of reconstituted basement membrane on the surface of the microporous filters. Matrigel reconstituted basement membrane (Collaborative Biomedical Products, Bedford, MA) was diluted to the desired concentration with ice-cold distilled water, applied to filters (12.5 μg/filter), dried for 30 min at 37°C and then dried overnight at room temperature. The in vitro invasion assay system is shown in Figure 2.2. The concentration of Matrigel used in the experiments was determined in a previous study in which we varied the amount of Matrigel placed on the filters (3.125, 6.25, 12.5, and 50 μg/filter) and determined the concentration that allowed a discriminating assay. The duration of incubation used in the experiments was determined similarly by varying the incubation time (12, 24, and 48 h) and identifying the time that allowed a discriminating assay (48 hours). MAT-LyLu and MLL cells (2 x 10^4 cells) in 0.15 ml 1640-RPMI medium containing 5% FCS were then added to the upper chamber in the presence of varying concentrations of GP (±) (0.0, 0.5, 1.0, and 2.0 μM) and cultured for 48 h. Then, the cells that penetrated the basement membrane and entered the bottom chamber, as well as the cells that remained in the upper chamber were collected by trypsinization and counted on a hemacytometer by trypan blue dye-exclusion method. The trypan blue dye-exclusion method was used to evaluate the cell viability. The invasion index was calculated as 100 x (number of cells in the bottom chamber) / (total number of cells in both chambers).
RT-PCR analysis.

RT-PCR was used to determine the nm23 mRNA expression levels in MAT-LyLu and MLL cells. Total RNA was isolated from MAT-LyLu and MLL cells by method as described in Chapter 1. Reverse transcription was performed as described in Chapter 1. PCR was performed by mixing 2 µl of RT product with 1.25 µl of MgCl₂ (50 mM), 2.5 µl of 10x PCR buffer, 0.2 µl of Taq polymerase (5 U/µl), and 0.3 µl of nm23 primers or 0.3 µl of β-actin primers in a total 25 µl. PCR for nm23 was run for 30 cycles of 95°C for denaturation for 45 sec, 65°C for annealing for 45 sec and 72°C for extension for 1 min. The 5’-primer for nm23 was 5’-ATG GCC AAC TGT GAG CGT ACC TTC-3’ and the 3’-primer was 5’-CTG TCA TTC ATA GAT CCA GTT CTG-3’. The 5’-primer for β-actin was 5’-ACC CAC ACT GTG CCC ATC TAG GA-3’ and the 3’-primer was 5’-GAT CCA CAT CTG CTG GAA GGT GG-3’. The final RT-PCR products (10 µl) were separated on a 1.2 % agarose gel containing ethidium bromide. The specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are presented as the ratio of nm23 to β-actin.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) for 4 culture wells. Using Minitab statistical software for Windows (Minitab Inc., State College, PA) performed 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

The invasiveness of parental MAT-LyLu and MLL cells

The invasiveness of parental MAT-LyLu and MLL cells was assessed by an in vitro invasion assay. After 48 hours of incubation in the in vitro invasion assay chamber system, MLL and MAT-LyLu cells in the upper chamber, as well as those that had passed through the filter and adhered to the bottom chamber, were harvested with trypsin and counted with a hemacytometer. The invasiveness of the cells is expressed as invasion index (calculated as described in the Materials and Methods). The results (Figure 2.3) showed that the MLL cells had a greater invasive index (24.4%) than the MAT-LyLu cells (15.0%), indicating a more invasive behavior for the MLL cells than for the parental MAT-LyLu cells (P<0.05). In addition, examination of the in vitro growth pattern of the two cell types by measuring their proliferation rates over a 72-hour culture period revealed that MLL cells have a greater growth rate than the parental MAT-LyLu cells (Figure 2.4). In vivo evidence of the tumorigenic potential of MLL cells is shown by the presence of a huge tumor mass on a Copenhagen rat that received a subcutaneous injection containing a suspension of MLL cells 15 days earlier (Figure 2.5, 2.6 and 2.7).

Expression of nm23 mRNA in MLL and MAT-LyLu cells

To elucidate the molecular basis of the observed differences in invasiveness of the MLL and MAT-LyLu cells, nm23 mRNA expression was assessed by RT-PCR analysis. Because the nm23 gene is a metastasis suppressor gene and the transfection of nm23 cDNA reduces the colonization and invasiveness of the human DU 145 prostate cancer
cell line in vitro (Lim et al., 1998), nm23 is a good marker to measure the metastatic ability of prostate cancer cells. Results of RT-PCR showed that the nm23 mRNA expression level in the parental MAT-LyLu cells was 1.5-fold greater than that in the MLL cells (Figure 2.8) (P<0.05). This finding is consistent with those generated from the in vitro invasion assays which showed greater invasiveness for the MLL cells which express lower levels of nm23 mRNA.

**Effects of GP (±) on the invasiveness of parental MAT-LyLu and MLL cells**

The effect of GP (±) on the invasiveness of MAT-LyLu and MLL cells was assessed by an in vitro invasion assay. GP (±) inhibited the invasiveness of both MAT-LyLu and MLL cells, as shown in Figure 2.9. GP (±) reduced the invasion indices of the MLL cells to 87.5%, 6.8% and 2.1% of the control at 0.5, 1.0 and 2.0 μM, respectively (P<0.05). Similarly, in MAT-LyLu cells, GP (±) reduced invasion indices to 86.7%, 6.0% and 1.1% of the control at 0.5, 1.0 and 2.0 μM, respectively (P<0.05). The inhibitory effect of GP (±) on the invasiveness of MAT-LyLu cells and MLL cells in the in vitro invasion assay system is consistent with the results of our previous in vivo study in which GP (±) reduced tumor growth and metastasis in MAT-LyLu-bearing Copenhagen rats (Chang et al., 1993).

**Effects of GP (±) on the nm23 mRNA expression of parental MAT-LyLu and MLL cells**

The effect of GP (±) on the nm23 mRNA expression of MAT-LyLu and MLL cells was assessed by RT-PCR analysis. Results of RT-PCR showed that GP (±) might elevate the nm23 mRNA expression of the MAT-LyLu cells to 3.7%, 15.3% and 22.8%
of the control at 0.5, 1.0 and 2.0 µM, respectively (P<0.05). Similarly, in MLL cells, GP (±) elevated nm23 mRNA expression to 7.2%, 9.8% and 21.8% of the control at 0.5, 1.0 and 2.0 µM, respectively (P<0.05) (Figure 2.10). Furthermore, the sensitivities of two cell types to GP (±) treatment were shown to be different. GP (±) at 1.0 µM is sufficient to significantly increase nm23 mRNA expression of MAT-LyLu cells, while in MLL cells, 2.0 µM of GP (±) are required to achieve a similar stimulation, suggesting that in nm23 mRNA expression, MLL cells to GP (±) treatment are less sensitive than MAT-LyLu cells. The stimulatory effects of GP (±) on the nm23 mRNA expression of MAT-LyLu cells and MLL cells were consistent with the results of our previous in vitro invasion assay in which GP (±) inhibited the invasiveness of both MAT-LyLu and MLL cells, as shown in Figure 2.9. This result suggests that the effects of GP (±) on the invasiveness of both MAT-LyLu cell and MLL cells in the in vitro invasion assays may be associated with the elevation of the nm23 mRNA expression. This finding provides the molecular basis of anti-metastasized ability of GP (±) against prostate cancer.

**Discussion**

Metastasis is one of the major causes of mortality in prostate cancer patients. Tumor invasion into the extracellular matrix and basement membrane is a crucial step in this process. Both in vitro and in vivo studies have demonstrated a correlation between tumor invasion and metastasis (Herrera-Gayol et al., 1995; Fisher et al., 1955). Thus, invasion plays an important role in the metastatic process of prostate cancer. The results of this study indicate that the MLL cells possess a nearly 1.6-fold greater ability to
penetrate a reconstituted basement membrane than the parental MAT-LyLu cells (24.4% vs 15.0% invasion index; Figure 2.3) as determined by an in vitro invasion assay. Thus, based on this assay, MLL cells exhibit a more invasive behavior than parental MAT-LyLu cells. This finding is consistent with our molecular analysis of the metastasis suppressor gene, nm23, which revealed a higher level of mRNA expression in the parental MAT-LyLu cell line than in the MLL cells (Figure 2.8). Reduced nm23 expression has been correlated with increased metastatic potential in human gastric carcinoma (Nakayama et al., 1993), prostate cancer (Konishi et al., 1993) and other cancer cell lines (Steeg et al., 1988; Bevilacqua et al., 1989; Yamaguchi et al., 1993). Several studies have shown that nm23 has a suppressive effect on the metastatic aggressiveness of melanoma and breast cancer cells in vivo (Leone et al., 1991; Leone et al., 1993). In addition, human prostate cancer cells transfected with nm23-H1 exhibited suppressed metastatic potential in vitro (Lim et al., 1998). Therefore, our results suggest that the observed difference in invasive ability in the two cell types (MLL and MAT-LyLu cells) may have a molecular basis in the expression of the nm23 metastasis suppressor gene. It is unclear however whether the MLL cells reflect a more invasive subpopulation of the original heterogeneous parental MAT-LyLu population, or whether the MLL cells acquired metastatic potential during the growth of the primary tumor. Furthermore, whether these differences between the two cell types in invasive ability and nm23 expression will be evident in vivo, in the form of differences in the number of metastatic foci in lungs and lymph nodes for instance, is not yet known.

GP (±) can serve as a potent chemotherapeutic agent against human androgen-dependent and -independent prostate disease. GP (±) has been shown to inhibit cell
growth and DNA synthesis, to induce TGFβ1 mRNA expression and protein secretion, and to block the cell cycle at the G0/G1 phase in human prostate cancer and benign prostatic hyperplastic cells (Shidaifat et al., 1996; Shidaifat et al., 1997). Our previous results have also shown that GP (±) can reduce in vivo tumor growth and incidence of lung and lymph node metastasis in MAT-LyLu-bearing Copenhagen rats (Chang et al., 1993). The results of this study show that GP (±), at the concentrations tested in this study, was equally effective in inhibiting the invasive behavior of both the parental MAT-LyLu and MLL cell lines. This result is consistent with the findings of our previous in vivo study in MAT-LyLu-bearing Copenhagen rats in which GP (±) inhibited metastasis (Chang et al., 1993). This result also indicates that GP (±) possesses the ability to inhibit cancer cells regardless of metastatic potential, a characteristic that demonstrates the potential value of GP (±) for the treatment of cancer patients including those with metastatic disease. Under our experimental conditions, the sensitivities of the two cell types to GP (±) with respect to in vitro invasive ability were not shown to be different. This finding may have been the result of the GP (±) concentrations selected for treatment. Perhaps, differences in sensitivities would be detected at GP (±) concentrations between 0.5 μM (which induced no changes in invasion indices) and 1.0 μM (which resulted in maximal inhibition). Furthermore, differences between the MLL and MAT-LyLu cells in sensitivity to GP (±) may be apparent in other parameters of cellular function and behavior, such as cell proliferation, oncogene expression, and tumor suppressor gene expression.

The mechanism by which GP (±) inhibits the invasive activity of cancer cells is unknown. In this study, we found that GP (±) can enhance the nm23 mRNA expression in
both MAT-LyLu and MLL cells. The sensitivities of two cell types to GP (±) treatment were shown to be different. GP (±) at 1.0 μM is sufficient to significantly increase nm23 mRNA expression of MAT-LyLu cells, while in MLL cells, 2.0 μM of GP (±) are required to achieve a similar stimulation, suggesting that in nm23 mRNA expression, MLL cells to GP (±) treatment are less sensitive than MAT-LyLu cells. It is not clear why the MLL cells are less sensitive to GP (±) than MAT-LyLu cells. Perhaps the cytosolic and mitochondrial enzymes of MLL cells are less responsive to GP (±) than those of MAT-LyLu cells. It seems that cancer cells with more invasive ability are more resistant to GP (±) treatment.

From the above result, we conclude that the anti-invasive ability of GP (±) on MAT-LyLu and MLL cells may be associated with the elevation of nm23 metastasis suppressor gene expression. It is known that nm23 gene inhibits the ability of DU145 prostate carcinoma cells to adhere to extracellular matrix (ECM) (Lim et al., 1998). Thus, a possible mechanism may involve interference with metastasis and invasion of prostate cancer cells through affecting on the expression of metastasis suppressor gene, nm23. The invasive inhibition of prostate cancer cells by GP (±) may be involved, at least partially, in the stimulation of nm23 gene expression. The stimulation of nm23 gene may be the molecular mechanism(s) of GP (±)-induced reductions in cancer cell invasive ability. However, other potential mechanisms of GP (±)’s antimitastatic activity cannot be excluded. For example, polyamines were proposed as critical mediators of tumor progression, including invasion and angiogenesis. Ornithine decarboxylase (ODC) is a key enzyme in polyamine synthesis and its overexpression was also linked to invasion (Weiss., 2000). Our laboratory showed that GP (±) significantly induced the expression of spermidine/spermine-N1-acetyltransferase (SSAT) mRNA, the major catabolic enzyme for polyamines in canine
prostate epithelial cells (Chang et al., 1997). Thus, it may speculate that the invasive inhibition of prostate cancer cells by GP (±) may be also mediated by inducing, at least partially, the expression of spermidine/spermine-N1-acetyltransferase (SSAT) which prevent accumulation of polyamines in prostate cancer cells. In addition, the possible mechanism of anti-metastatic effects of GP (±) may be also associated with inhibiting the activity of protein kinase C (PKC). It is known that PKC play a critical role in the metastasis of cancer cells and PKC expression correlates with metastatic potential (Debies et al., 2001). Kiley et al. showed that the delta isoform of PKC was upregulated and activated in metastatic mammary tumors (Kiley et al., 1999a; Kiley et al., 1999b). GP (±) was demonstrated to inhibit the PKC activity in cultured rat spermatocytes (Teng., 1995). Thus, the antimetastatic ability of GP (±) on the prostate cancer cells may be related to the inhibition of PKC activity.

The availability of both MAT-LyLu and MLL cells provides novel and valuable tools for investigating the biology, behavior, and response of metastatic cells to chemopreventive/therapeutic agents in both \textit{in vitro} and \textit{in vivo} models of metastasis. Currently, we have already collected and cultured the invasive subpopulations of MAT-LyLu and MLL cells that penetrated the reconstituted basement membrane in the invasion assay chamber. These cells should provide a useful tool for the study of the invasive behavior of cancer cells both \textit{in vitro} and \textit{in vivo}. The anti-metastatic effect of GP (±) could be mediated by multiple mechanisms (nm23 metastasis suppressor gene blocking PKC activity, and polyamines metabolism). In light of these finding, GP (±) could be a potent anti-metastatic agent against prostate cancer. Thus, the ability of GP (±) to induce nm23 metastasis suppressor gene possesses significant implication for
clinical treatment of prostate cancer. GP (±) may be used alone or in combination with other therapeutic agents as an anti-metastatic agent for prostate cancer patients with metastasis.
Figure 2.2. **In vitro invasion assay system.** The 24-well Transwell™ system was used for in vitro invasion assays. Cell culture inserts (6.5 mm diameter) were converted into invasion chambers by applying a layer of basement membrane on the surface of the microporous filters (8 µm pore size). Matrigel™ reconstituted basement membrane was diluted to the desired concentration with ice-cold distilled water and then applied to filters. The amount of Matrigel™ used in the experiments was 12.5 µg/filter.
Figure 2.2. In vitro invasion assay system
Figure 2.3. Comparison of the invasiveness of parental MAT-LyLu and MLL cells

The invasiveness of parental MAT-LyLu and MLL cells assessed by an in vitro invasion assay. MAT-LyLu and MLL cells (2 x 10⁴ cells) in 0.15 ml 1640-RPMI medium containing 5% FCS were then added to the upper and cultured for 48 h. Then, the cells that penetrated the basement membrane and entered the bottom chamber, as well as the cells that remained in the upper chamber were collected by trypsinization and counted on a hemacytometer by trypan blue dye-exclusion method. The trypan blue dye-exclusion method was used to evaluate the cell viability. The penetrated cell number of MAT-LyLu and MLL cells is 3533 cells and 6466 cells respectively. The invasion index was calculated as: 100 x (number of cells in the bottom chamber) ÷ (total number of cells in both chambers). The invasion index in MAT-LyLu and MLL cells is 15.0% vs 24.4% respectively. Each bar represents the Mean ± SD of 3 replicate wells. Asterisks indicate means that are significantly different from their respective control means. A probability (p) of less than 0.05 was considered statistically significant.
Figure 2.3

- N=3 (well)
- Mean±SD
- *P<0.05
Figure 2.4. Comparison of the growth of MAT-LyLu and MLL cells. The growth of MAT-LyLu and MLL cells assessed by cell number calculation. Cell numbers were determined using a hemacytometer and the trypan blue dye-exclusion method at different times after treatment (0, 12, 24, 36, 48, 60, and 72 h). The trypan blue dye-exclusion method was used to evaluate the cell viability. The cells were examined in a counting chamber with a light microscope. Only viable cells were recorded. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 2.4

MAT-LyLu DT=10.4 (hours)
MLL DT=8.57 (hours)

N=4 (well)
MeansSD
Figure 2.5. Phase-contrast micrograph of cultured MLL cells. MLL cells from metastasized lungs of MAT-LyLu cells bearing Copenhagen rats were isolated as described in the Materials and methods section. MLL cells were cultured in RPMI-1640 medium containing 5% FCS (400x).
Figure 2.5
Figure 2.6. A male Copenhagen rat with a large MLL cell tumor. The photograph shows a male Copenhagen rat with a large MLL cell tumor approximately 15 days after subcutaneous implantation of $0.75 \times 10^6$ MLL cells.
Figure 2.6
Figure 2.7. MLL cell tumor from a male Copenhagen rat. The photograph shows the excised tumor (approximately 30 g) from the rat.
Figure 2.7
Figure 2.8. The expression of nm23 mRNA in parental MAT-LyLu and MLL cells. The expression of nm23 mRNA in MAT-LyLu and MLL cells assessed by RT-PCR analysis. Total RNA was isolated from cultured MAT-LyLu and MLL cells and used for RT-PCR analysis of nm23 mRNA expression. β-Actin was used as an internal loading control. The results are expressed as the relative expression ratios of nm23 to β-actin. Each bar represents the mean ± SD of 3 replicate samples. An image of ethidium bromide-stained PCR products separated in an agarose gel from a representative experiment is shown. The asterisk indicates a statistically significant difference between the two means. A probability (p) of less than 0.05 was considered statistically significant.
Figure 2.8
Figure 2.9. Effects of GP (±) on the invasiveness of parental MAT-LyLu and MLL cells. Effects of GP (±) treatment on the invasiveness of parental MAT-LyLu and MLL cells assessed by an in vitro invasion assay. MAT-LyLu and MLL cells were treated with 0, 0.5, 1.0, and 2.0 μM of GP (±) for 48 h after seeding into the upper chambers of the Transwell™ system. After 48 hours of incubation, cells remaining in the upper chamber and those that had passed through the filter and adhered to the bottom chamber were harvested by trypsin and counted with a hemacytometer. Invasion indices were calculated as described in the Materials and Methods as a measure of invasiveness. Each bar represents the Mean±SD of 3 replicate wells. Asterisks indicate means that are significantly different from their respective control means. A probability (p) of less than 0.05 was considered statistically significant.
Figure 2.9

Cell Penetration (%) vs. Gossypol Concentration (µM)

- MLL
- MAT-LyLu

N=3 (well)
Mean±SD
*P<0.05
Figure 2.10. Effects of GP (±) on the nm23 mRNA expression of parental MAT-LyLu and MLL cells. The expression of nm23 mRNA in MAT-LyLu and MLL cells assessed by RT-PCR analysis. MAT-LyLu and MLL cells were treated with 0, 0.5, 1.0, and 2.0 μM of GP (±) for 24 h. After 24 hours of incubation, total RNA was isolated from cultured MLL and MAT-LyLu cells and used for RT-PCR analysis of nm23 mRNA expression. β-Actin was used as an internal loading control. The results are expressed as the relative expression ratios of nm23 to β-actin. Each bar represents the Mean±SD of 3 replicate samples. An image of ethidium bromide-stained PCR products separated in an agarose gel from a representative experiment is shown. The asterisk indicates a statistically significant difference between the two means. A probability (p) of less than 0.05 was considered.
Figure 2.10

Figure 2.10

**MAT-LyLu**
- β-actin (593 bp)
- nm23 (460 bp)

**MLL**
- β-actin (593 bp)
- nm23 (460 bp)

Figure 2.10

![Graph showing relative expression ratio of nm23:actin with Gossypol concentration (µM) and N=3, Mean±SD, *P<0.05](image)

**N=3**
**Mean±SD**

*P<0.05
CHAPTER 3

O₂ CONSUMPTION AND CO₂ PRODUCTION IN CULTURED PROSTATE CANCER CELLS, CULTURED PROSTATE CANCER TISSUE, AND CULTURED METASTATIC LUNG TISSUE

Abstract

In an attempt to measure the respiration in prostate cancer tissue, tumor was allowed to grow in Copenhagen rats following inoculation of MAT-LyLu prostate tumor cells. A computerized O₂/CO₂ respirometer (Micro-Oxymax respirometer) was used to measure O₂ consumption and CO₂ production by normal prostate tissue, prostate tumor tissue, normal lung tissue and metastatic lung tissue in explant culture. The results showed that O₂ utilization and CO₂ production by the prostate tumor tissue and malignant lung tissue are distinctly higher than the corresponding normal tissue. It suggests that Micro-Oxymax respirometer has some potential in distinguishing tumor tissue from the normal tissue by their pattern of respiration.
Racemic gossypol \([\text{GP (\pm)}]\) is a potent antifertility agent contained in cottonseed and other parts of cotton plants. We have shown that \(\text{GP (\pm)}\) inhibits in vitro growth of Dunning rodent prostate cancer cell (MAT-LyLu), PC3, MCF-7 and primary cultured human prostate cells. We have also shown that \(\text{GP (\pm)}\) has some antiproliferative and antimetastatic effects on transplanted prostate tumor in Copenhagen rats. In this experiment, \(\text{GP (\pm)}\)’s effects on the proliferation of MAT-LyLu cells and PC3 cells were assessed by thymidine incorporation assay. \(\text{GP (\pm)}\)’s effects on \(\text{O}_2\) consumption and \(\text{CO}_2\) production of MAT-LyLu cells and PC3 cells were monitored using Micro-Oxymax respirometer. The \(\text{GP (\pm)}\)’s effects on oxidative phosphorylation were determined by succinic dehydrogenase assay. The result showed that after MAT-LyLu cells and PC3 cells exposed to \(\text{GP (\pm)}\) for 24 h, \(\text{GP (\pm)}\) at the concentration of 1.0 \(\mu\text{M}\) caused reductions in DNA synthesis of MAT-LyLu cells and PC3 cells at the different time after treatment (24, 28 and 32 h) respectively. \(\text{GP (\pm)}\) at the concentration of 2.0 \(\mu\text{M}\) decreased significantly DNA synthesis of MAT-LyLu cell and PC3 cells at the different time after treatment (24, 28 and 32 h) respectively. A significant reduction in \(\text{O}_2\) consumption and \(\text{CO}_2\) production in \(\text{GP exposed cells}\) was also observed. \(\text{GP (\pm)}\) at the concentration of 2.0 \(\mu\text{M}\) significantly decreased \(\text{O}_2\) consumption and \(\text{CO}_2\) production in MAT-LyLu cells and PC3 cells at the different time after treatment (24, 28 and 32 h) respectively. \(\text{GP (\pm)}\) at the concentration of 1.0 \(\mu\text{M}\) did not significantly inhibit \(\text{O}_2\) consumption and \(\text{CO}_2\) production of MAT-LyLu cells and PC3 cells. By succinic dehydrogenase analysis, we observed that \(\text{GP (\pm)}\) might inhibit the activity of mitochondrial succinic dehydrogenase of MAT-LyLu cells and PC3 cells at the concentration of 1.0 \(\mu\text{M}\) and at the concentration of 2.0 \(\mu\text{M}\) respectively. The experimental results suggest that respirometric method has a
certain potential for the study of cellular respiration in culture and that GP (±) inhibits prostate tumor cell respiration and cellular proliferation. The inhibitory action of GP (±) on O\(_2\) consumption and CO\(_2\) production of prostate tumor cells may be due to inhibition of oxidative phosphorylation as a result of inhibiting mitochondrial succinic dehydrogenase in prostate tumor cells.

**Introduction**

Tumor tissue, when grown *in vivo*, exhibits a hypoxic state because of multiple pathophysiological interactions. It has been suggested that structural and functional abnormalities in tumor vessels lead to decreased O\(_2\) delivery to the tumor tissue (Dunn., 1997). Moreover, the cancer tissue consumes more O\(_2\) because of the increased proliferation of the cells. Thus, cancer tissue exhibits hypoxia because of the increased O\(_2\) demand by cancer cells and decreased O\(_2\) supply caused by abnormalities in tumor blood vessels (Dunn., 1997). The proliferation of tumor cells depends on the supply of nutrients and O\(_2\). Respiration, the consumption of O\(_2\) and production of CO\(_2\) is one of the key physiological function for cell survival and proliferation.. Cancer cells, like normal cell, derive their cellular energy from ATP through mitochondrial respiration and oxidative phosphorylation (Benz et al., 1987). Cancer cells are characterized by a high rate of glycolysis under aerobic condition (Beckner et al., 1990) and have higher sensitivity to antimitochondrial drugs than normal cells (Wilkie et al., 1979). The time course of glycolytic CO\(_2\) production of malignant tumor cells correlated directly with their motility (Beckner et al., 1990).
Racemic gossypol [GP (±)], a polyphenolic compound naturally occurring in cottonseed, has gained interest as a male antifertility agent and an anticancer agent (Kalla et al., 1985; Ranga et al., 1990; Lin et al., 1992). GP (±) can inhibit the in vitro growth of MCF-7, and PC3 cells (Ligueros et al., 1997; Shidaifat et al., 1996), as well as the in vivo proliferation and metastasis of androgen-independent prostate cancer cells, MAT-LyLu cell line, after implantation into Copenhagen rats (Chang et al., 1993). GP (±) inhibits the invasiveness of MAT-LyLu cells and MAT-LyLu cells from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats (Jiang et al., 2000). Early studies in our laboratory showed that GP (±) arrested the cell cycle of PC3 cells, human BPH cells and stromal cells derived from human breast adipose tissues at the G1/S phase by inducing transforming growth factor β1 production (Shidaifat et al., 1996; Shidaifat et al., 1997; Zhang et al., 1998). Other group also found that GP (±) arrested the cell cycle of MCF-7 cells at the G1/S phase by decreasing Rb expression, Rb phosphorylation and cyclin D1 expression (Ligueros et al., 1997). In addition, our laboratory also found that there was a significant increase of wild type p53 protein production in GP (±)-treated MCF-7 cell (Sugimoto et al., 1997). GP (±) has the abilities of interfering with a variety of enzymes including hexokinase, lactate dehydrogenase, cytochrome oxidase, and succinic dehydrogenase, which are involved in energy production (Ueno et al., 1988; Lee et al., 1982; Burgos et al., 1985; Ferguson et al., 1959; Lin et al., 1987). GP (±) has also exhibited antimitochondrial activity and depressed oxygen uptake in the guinea-pig Keratinocyte (Bugeja et al., 1988). The antimitochondrial activity of GP (±) includes the uncoupling of oxidative phosphorylation, inhibition of the mitochondrial respiratory chain and inhibition of ATP production (Benz et al., 1987; Bugeja et al., 1988).
antitumor ability of GP (±) may be due to its ability of uncoupling tumor mitochondria (Benz et al., 1990). The blockade of oxygen metabolism of mitochondria may result in the change in \( O_2 \) consumption and \( CO_2 \) production of malignant cells. Thus, \( O_2 \) consumption and \( CO_2 \) production of cells are a valuable indicator in determining the metabolic activity of tumor cells. The present study has been designed to establish whether cancer tissue respiration is measurably different from respiration of non-malignant tissue. In addition, we have attempted to examine the effects of GP (±) on the \( O_2 \) consumption and \( CO_2 \) production in prostate cancer cell lines (MAT-LyLu and PC3). We measured change of \( O_2 \) and \( CO_2 \) gas levels of MAT-LyLu and PC3 cells by Micro-Oxymax respirometer. Micro-Oxymax respirometer is specially designed to monitor extremely low levels of oxygen consumption with an electrochemical oxygen sensor and low levels of carbon dioxide production using infra-red sensor. Micro-Oxymax respirometer performs measurements on a closed system where the gas mixture in the test chamber is pumped through the gas sensors and then returned to the test chamber. The percentage of \( O_2 \) and \( CO_2 \) gas levels of the test chamber environment are measured periodically and the changes in the levels are used to compute the \( O_2 \) consumption and \( CO_2 \) production. Because \( O_2 \) consumption and \( CO_2 \) production are related to metabolic process, succinic dehydrogenase (SDH) is an enzyme that catalyzes the dehydrogenation of succinate to fumarate in the tricarboxylic (Krebs) cycle. It plays a critical role in cellular bioenergetics in where Krebs cycle involved in energy generation and is used to produce precursors for the biosynthesis of amino acids, purines, pyrimidines and vitamins (Chalmers et al., 1992). The measurement of the activity of SDH of the Krebs’ cycle has been considered as an indicator of oxidative metabolic capacity (Fattoretti et al., 1998).
SDH may be an appropriate target for an anticancer agent. Douglas’s research team showed that the histochemical activity of SDH is present in primary epithelial cells from rat ventral prostate, but not in stromal cells (Douglas et al., 1980). Ferguson’s group found that GP (±) almost completely inhibited SDH and cytochrome oxidase activity from liver homogenate of mature hen, while endogenous respiration and xanthine oxidase activity were reduced to less than half of original value (Ferguson et al., 1959). Our laboratory also observed that GP (±) had higher affinity to mitochondrial membrane and inhibited mitochondrial enzymes (Lin et al., 1992). Thus, in order to explore feasibility of O₂ consumption and CO₂ production of tumor tissue as a marker of early diagnosis, we measured the O₂ consumption and CO₂ production in a variety of prostate tissues, lung tissues, and prostate cells. In addition, we have also attempted to examine inhibitory effects of GP (±) on the growth and cellular respiration of prostate cancer cells and the possible mechanism of inhibitory effects of GP (±) on cellular respiration of prostate cancer cells.

**Materials and methods**

**Reagent**

Racemic gossypol [GP (±)] (Sigma Chemical Co., St. Louis, MO) was dissolved in 100% ethanol to make a 10 mM stock solution. Treatment solutions were prepared by the dilution of stock solution in RPMI-1640 medium (GibcoBRL) with 5% fetal calf serum (FCS).
Animals, and implantation of prostate tumor tissues

The rat prostate tumor tissue (MAT-LyLu) was used in this study. Male Copenhagen rats (120-140 days of age) with body weights ranging from 200 to 250 g were randomly selected as MAT-LyLu prostate cancer cell recipients. Rats were divided into two groups. The transplantation of MAT-LyLu cells was started after five days of accommodation. The viable MAT-LyLu cells (0.75 x 10^6/rat) suspended in hank’s balanced salt solution (HBSS) were injected subcutaneously into the flank of rats in group one while the rats in group two were treated with HBSS as control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 15th day while the remaining animals in group two were killed on 26th day. Following tumor cell incubation, the prostate tumor tissue and lung from tumor bearing rats and normal prostate tissue and lung from HBSS treated rats were dissected out and used for the evaluation of metabolic respiration.

Measurement of O_2 consumption and CO_2 production of tissues

250 mg of each of lung, prostate tumor tissue, and normal prostate tissue were placed separately in a cultured flask containing 25 ml RPMI-1640 medium (GibcoBRL). RPMI-1640 medium were supplemented with 5% FCS and an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (GibcoBRL). The cultured flasks were transferred to an incubator (37°C, 5% CO_2 and 95% air) attached to the Micro-Oxymax respirometer (Columbus Instruments, Columbus, OH). The data on O_2 consumption and CO_2 production at different times of incubation (0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 h) were automatically recorded by a computer.
The diagrammatic representation of Micro-Oxymax respirometer was shown in Figure 3.1. The instrument measures changes in O$_2$ and CO$_2$ concentrations in the culture flasks over time. It operates on the principle of using gas sensors to measure the change in O$_2$ and CO$_2$ concentration in the head space of the measuring flasks. The operation of the instrument is based on the principle that the consumption or production on two consecutive readings with the volume of the gas. The measuring process consists of measuring the gas concentration in the sensors and in each measuring chamber. Within the instrument, the air from the chamber is circulated through the sensors and sensor is allowed to measure and after which the air is returned to the chamber. A small amount of air in the sensors mixes with the air in the chamber and helps in keeping the sample at consistent O$_2$ and CO$_2$ levels. If the level of gas drops below or exceeds a user-defined or system-defined level, the system will flush the chamber with fresh air from the refresh port. Basically, the system looks at the O$_2$ and CO$_2$ concentration in the consecutive air supply readings to measure any drift in the sensor output. The measured value for the drift in the sensors is used to correct the consumption and production results.

*Cell culture of prostate cancer cell lines*

The prostate cancer cell line, MAT-LyLu cells and PC3 cells were cultured as previously described in *Material and Methods* section of Chapter 1.
Fig. 3.1. Diagramatic representation of Micro-Oxymax Respirometer with electrochemical O₂ sensor.
Thymidine incorporation assay

To measure the cell proliferation of MAT-LyLu cells and PC3 cells, 2x10^4 MAT-LyLu cells or PC3 cells per well were cultured in 24-well respectively. After 24 h, the cells were treated with the different concentrations of GP (±) (0.0, 1.0, and 2.0 μM) and the ³H-thymidine incorporation of cells at different times (24, 28, and 32 h) after treatment were measured. The procedure followed those described in Materials and Methods section of Chapter 1.

Measurement of O₂ consumption and CO₂ production of MAT-LyLu cells and PC3 cells

Approximately, 1.5x10^6 viable PC3 and MAT-LyLu cells were cultured in 25 cm² flask containing 5 ml RPMI-1640 medium with 5% FCS. After 24 h, the cells were treated with GP (±) at 0, 1.0, 2.0 μM, flasks were transferred to an incubator attached to the Micro-Oxymax respirometer (Columbus Instruments, Columbus, OH). The data on O₂ consumption and CO₂ production at different times after treatment (0, 4, 8, 12, 16, 20, 24, 28, and 32 h) were automatically recorded by a computer.

The instrument measures changes in O₂ and CO₂ concentrations in the culture flasks over time. The operation and measuring process of instrument was described in measurement of O₂ consumption and CO₂ production of tissue section in Materials and methods of this chapter.
The measurement of the activity of mitochondrial succinic dehydrogenase of MAT-LyLu cells and PC3 cells

2x10^6 MAT-LyLu cells or PC3 cells were cultured in 75 cm² flasks and maintained in RPMI-1640 supplemented with 5% FCS. Mitochondria were isolated by the method as described by Berner et al with modification (Berner et al., 1999). When cells are 80% confluent, MAT-LyLu cells or PC3 cells were scraped off from flasks and homogenizes in 0.25 M sucrose buffer containing 10 mM HEPES (Fisher Scientific, Pittsburgh, PA) and were sonicated (Sonicator W-380) by three 20-s bursts maintained at 4° C respectively. The homogenate was centrifuged at 4500 xg for 10 min at 4° C to sediment cell membrane and unbroken cells. Supernatant was centrifuged at 16,000 xg for 25 min at 4° C. The resulting pellet was suspended in 0.25 M sucrose buffer containing in 10 mM HEPES (Fisher Scientific) and the mitochondrial homogenate solution containing 200 μg/ml protein for succinic dehydrogenase assay. The mixture solution contained 0.2 M phosphate buffer, 1% (w/v) bovine serum albumin (BSA), 0.005 M potassium cyanide (KCN), and 0.00025M Dichlorophenolindophenol (DCPIP) in the presence of varying concentrations of GP (±) (0.0, 1.0, 2.0 μM). 0.5 ml 0.6 M sodium succinate (all components purchased from Sigma Chemical Co.) and 0.5 ml mitochondrial fraction (100 μg/ml) were added to this mixture. The decrease in absorbance at 600 nm was monitored for 7 min. Enzyme activity was expressed as percentage of succinic dehydrogenase activity.
**Statistical analysis**

Data were expressed as the mean ± standard deviation (SD) for 4 cultures well. Using Minitab statistical software for Windows (Minitab Inc., State College, PA) performed data 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**

*Comparison of the O₂ consumption and CO₂ production of normal prostate and prostate tumor (MAT-LyLu) tissue in explant culture*

It is known that the cells in cancer tissue multiply faster in an uncontrolled fashion. So, for maintaining their high activity, the cancer cells consume more O₂ and produce more CO₂ (Beckner et al., 1990). To determine whether Micro-Oxymax respirometer can detect any change in O₂ consumption and CO₂ production in prostate tumor tissue, we used mature Copenhagen rats for this experiment. Rats in group one received viable MAT-LyLu tumor cells (0.75x10⁶/rat) while rats in group two received same volume of vehicle (HBSS). The O₂ consumption and CO₂ production of normal prostate and prostate tumor (MAT-LyLu) tissue in explant culture were automatically evaluated by the Micro-Oxymax respirometer. The O₂ consumption and CO₂ production in normal prostate and prostate tumor (MAT-LyLu) tissue on 15th day after tumor cells or vehicle injection was examined as shown in Figure 3.2 and Figure 3.3 respectively. The results showed that O₂ consumption and CO₂ production in normal prostate and
prostate tumor (MAT-LyLu) tissue is more or less similar during first 8 hours of tissue culture but following this period, the O\textsubscript{2} consumption and CO\textsubscript{2} production in tumor tissue increased more rapidly than in the normal prostate tissue. At 40 hours of culture, the O\textsubscript{2} consumption of prostate tumor (MAT-LyLu) tissue showed 4 fold higher than that of normal prostate tissue, while the CO\textsubscript{2} production of prostate tumor (MAT-LyLu) tissue showed 2.2 fold higher than that of normal prostate tissue. The increase in O\textsubscript{2} consumption of prostate tumor (MAT-LyLu) tissue is parallel to the increase of CO\textsubscript{2} production of that tissue. In this experiment, the value of CO\textsubscript{2} production is smaller than that of O\textsubscript{2} consumption. This difference may be due to the partial dissolution of CO\textsubscript{2} gas in the culture medium. These results indicate that tumor tissues consume more O\textsubscript{2} and produce more CO\textsubscript{2} production than normal prostate tissues.

*Comparison of the O\textsubscript{2} consumption and CO\textsubscript{2} production of normal lung tissue and metastatic lung tissue in explant culture*

MAT-LyLu cells are recognized to have been originally derived from the prostate and to be able to metastasize to, and develop foci and proliferation in the lung. The present study has attempted to test whether respiration of metastatic lung tissue from tumor bearing rat is measurably different from respiration of normal lung tissue. The O\textsubscript{2} consumption and CO\textsubscript{2} production of normal lung, metastatic lung, and prostate tumor (MAT-LyLu) tissue in explant culture were automatically evaluated by the Micro-Oxymax respirometer. In this experiment, the O\textsubscript{2} consumption and CO\textsubscript{2} production of the prostate tumor (MAT-LyLu) tissue serve as control. The O\textsubscript{2} consumption and CO\textsubscript{2} production in normal lung and metastatic lung tissue on 15th day and 26th day after
tumor cells or vehicle injection was examined respectively. The results showed that the lung tissue collected from both tumor bearing rats and normal rats on 15th day after tumor cells or vehicle injection showed similar pattern of O$_2$ consumption and CO$_2$ production in explant culture as shown in Figure 3.4 and Figure 3.5 respectively. This result suggests that Micro-Oxymax respirometer could not distinguish difference of O$_2$ consumption and CO$_2$ production between lung tissue from normal rat and metastatic lung from tumor bearing rat during 15th day of tumor cell injection. The lung tissue from tumor bearing rat on 26th days after tumor cells or vehicle injection showed higher O$_2$ consumption and CO$_2$ production than the lung from normal rats as shown in Figure 3.6 and Figure 3.7. This result indicates that Micro-Oxymax respirometer could distinguish the difference of O$_2$ consumption and CO$_2$ production between lung tissue from normal rat and metastatic lung from the tumor bearing rat on 26th day after tumor cell injection.

**Effects of GP (±) on the proliferation of MAT-LyLu cells**

The effects of GP (±) on the growth of malignant prostate cancer (MAT-LyLu) cells at different times were determined by thymidine incorporation assay. Cells were treated with increasing concentrations of GP (±) (0.0, 1.0, and 2.0 μM) for 24, 28, and 32 h. The results showed that GP (±) significantly inhibited the DNA synthesis of human cancer-MAT-LyLu cell as shown in Figure 3.8. GP (±) at the concentration of 1.0 μM caused reductions in DNA synthesis by 28.2%, 40.7%, and 56.7% at the different times (24, 28, and 32 h) after GP (±) treatment respectively, while GP (±) at the concentration of 2.0 μM caused reductions in DNA synthesis by 40.7%, 56.3%, and 63.5% of MAT-
LyLu cells at the different times (24, 28, and 32 h) respectively. GP (±) decreased the DNA synthesis of MAT-LyLu cells in dose-dependent manner. The growth inhibition of GP (±) in the MAT-LyLu cells gradually increased with increasing of time of GP (±) treatment. These results are consistent with that GP (±) inhibits the growth of human prostate cancer cells (Shidaifat et al., 1996).

Effects of GP (±) on the O$_2$ consumption and CO$_2$ production of MAT-LyLu cells

The effects of GP (±) on the oxidative catabolism of MAT-LyLu cells were assessed by measuring O$_2$ consumption and CO$_2$ production. The O$_2$ consumption and CO$_2$ production of MAT-LyLu cells were automatically evaluated by the Micro-Oxymax respirometer. GP (±) inhibited the O$_2$ consumption and CO$_2$ production of MAT-LyLu cells as shown in Figure 3.9 and Figure 3.10 respectively. The results showed that GP (±) at the concentration of 2.0 µM caused a significant decrease in O$_2$ consumption by 10.5%, 12.0% and 13.0% compared to control at the different time after treatment (24, 28 and 32 h) (P<0.05). This reduction is parallel to the inhibition of CO$_2$ production of MAT-LyLu cells in a dose-dependent manner. While GP (±) at the concentration of 2.0 µM decreased CO$_2$ production by 33.2%, 33.8% and 34.3% compared to control at the different time after treatment (24,28 and 32 h) (P<0.05). MAT-LyLu cells with the treatment of 1.0 µM GP (±) had no significant effects on O$_2$ consumption and CO$_2$ production. These results are consistent with that GP (±) inhibits the DNA synthesis of MAT-LyLu cells.
Effects of GP (±) on the succinic dehydrogenase activity of the mitochondrial of MAT-LyLu cells.

Since Succinic dehydrogenase (SDH) is of critical importance in cellular bioenergetics, especially in Krebs cycle which is involved in energy generation and the production of precursors for the biosynthesis of amino acids, purines, pyrimidines and vitamins (Chalmers et al., 1992), the antitumor ability of GP (±) may relate to its ability to uncouple tumor mitochondria (Benz et al., 1990). To elucidate the mechanism of action of GP (±) on the O₂ consumption and CO₂ production, the effects of GP (±) on mitochondrial oxidation reaction was assessed by succinic dehydrogenase assay. In the initial studies, we established time course for SDH activities of mitochondrial sonicated preparation to reach equilibrium. Figure 3.11 demonstrated that under the conditions employed, equilibrium was read in 7 min in the presence or absence of GP (±) in the preparation. Results of enzyme assay showed that GP (±) significantly inhibited the activity of mitochondrial SDH to 40.6% of control at the concentration of 1.0 μM and 3.4% of control at the concentration of 2.0 μM respectively (P<0.05). This reduction is parallel to the inhibition of O₂ consumption and CO₂ production of MAT-LyLu cells in a dose-dependent manner. Furthermore, the finding also showed that the mitochondrial SDH in MAT-LyLu cells to GP (±) treatment was more sensitive than O₂ consumption and CO₂ production in MAT-LyLu cells. The result suggests that the effects of GP (±) on the O₂ consumption and CO₂ production of MAT-LyLu cells may be relate to the inhibition of mitochondria SDH activity of MAT-LyLu cells.
Effects of GP (±) on the proliferation of PC3 cells

The growth of cells depends on the supply of nutrients and O₂. Cell respiration, the consumption of O₂ and production of CO₂, play important roles in the proliferation and function of cells. In order to test whether other prostate cancer cells, like MAT-LyLu cells, have a same response pattern to GP (±) treatment, the effects of GP (±) on the growth and metabolic respiration of malignant prostate cancer (PC3) cells were examined by thymidine incorporation assay and Micro-Oxymax respirometer respectively. Cells were treated with increasing concentrations of GP (±) (0.0, 1.0, and 2.0 μM) for 24, 28, and 32 hours. The results showed that GP (±) significantly inhibited the DNA synthesis of human cancer-PC3 cell as shown in Figure 3.12. GP (±) at the concentration of 1.0 μM caused reductions in DNA synthesis by 25.6%, 54.6%, and 81.25% at the different times (24, 28, and 32 h) after GP (±) treatment respectively, while GP (±) at the concentration of 2.0 μM caused reductions in DNA synthesis by 78.57%, 81.44%, and 83.72% of PC3 cells at the different times (24, 28, and 32 h) respectively. GP (±) decreased the DNA synthesis in PC3 cells in dose-dependent manner and resulted in a significant reduction in DNA synthesis at the concentration of 1.0 μM at 28 and 32 h and 2.0 μM at 24, 28, and 32 h respectively (P<0.05). These results are consistent with the growth inhibition of GP (±) in MAT-LyLu cells.

Effects of GP (±) on the O₂ consumption and CO₂ production of PC3 cells

In previous experiment, we have already confirmed that GP (±) could perturb cellular respiration of MAT-LyLu cells. In this experiment, we test whether GP can affect the oxidative catabolism of PC3 cells. The effects of GP (±) on the oxidative
catabolism of PC3 cells were assessed by measuring O$_2$ consumption and CO$_2$ production. The O$_2$ consumption and CO$_2$ production of PC3 cells were automatically evaluated by the Micro-Oxymax respirometer. GP (±) inhibited the O$_2$ consumption and CO$_2$ production of PC3 cells as shown in Figure 3.13 and Figure 3.14 respectively. The results showed that GP (±) at the concentration of 2.0 μM caused a significant decrease in O$_2$ consumption by 6.4%, 6.9% and 7.9% compared to control at the different time after treatment (24, 28 and 32 h) (P<0.05). This reduction is parallel to the inhibition of CO$_2$ production of PC3 cells in a dose-dependent manner. While GP (±) at the concentration of 2.0 μM decreased CO$_2$ production by 37.5%, 42.4% and 44.7% compared to control at the different time after treatment (24, 28 and 32 h) (P<0.05). PC3 cells with the treatment of 1.0 μM GP (±) had no significant effects on O$_2$ consumption and CO$_2$ production. These results are consistent with the respiration inhibition of GP (±) on MAT-LyLu cells. These results provide the evidence that GP (±) can interfere with the cellular respiration of prostate cancer cells. It is not clear whether the effects of GP (±) on the O$_2$ consumption and CO$_2$ production of MAT-LyLu and PC3 cells are direct or secondary to the inhibition of cell proliferation.

*Effects of GP (±) on the succinic dehydrogenase activity of the mitochondrial of PC3 cells.*

In above experiment, we observed that the inhibitory effect of GP (±) on the oxygen respiration of prostate cancer cells (PC3). To elucidate the mechanism of action of GP (±) on the O$_2$ consumption and CO$_2$ production, we used extracted mitochondria from PC3 cells to evaluate the mechanism of action of GP (±) on O$_2$ consumption and
CO₂ production. Mitochondrial oxidation reaction of PC3 cells was assessed by succinic dehydrogenase assay. From succinic dehydrogenase assay, we observed that GP (±) significantly inhibited the activity of mitochondrial SDH to 31% of control at the concentration of 1.0 μM and 15% of control at the concentration of 2.0 μM respectively (P<0.05) (Figure 3.15). This reduction is parallel to the inhibition of O₂ consumption and CO₂ production of PC3 cells in a dose-dependent manner. In addition, we also found that the mitochondrial SDH in PC3 cells to GP (±) treatment was more sensitive than O₂ consumption and CO₂ production in PC3 cells. It is well known that GP (±) have higher affinity to mitochondrial membrane and inhibit mitochondrial enzymes (Lin et al., 1992). Our result suggests that the inhibitory effects of GP (±) on the O₂ consumption and CO₂ production in PC3 cells may be mediated by inhibiting mitochondria SDH activity of PC3 cells.

**Discussion**

The diagnosis of cancer at an early stage is a world wide problem. Prostate cancer is the most common malignancy in American men and appears to be a multifactoral neoplasm (Paganini-Hill et al., 1988; Parker et al., 1997). For reason yet unclear, cancer in the prostate continues to increase each year and over 50% of the patients have metastatic disease at the time when they are diagnosed of prostate cancer. However, diagnosis of prostate cancer at an early stage is difficult because it usually develops without symptoms. Thus, it is necessary to develop a precise method to detect the presence of the early prostate cancer. Research has shown that O₂ consumption in
LNCaP prostate tumor cells is increased by treatment with synthetic analog of androgen (Ripple et al., 1997). It is known that the growth of prostate tumor tissue in culture system is associated with higher consumption of O₂ and higher production of CO₂ (Dunn et al., 1997). Thus, the data on O₂ consumption of cells or tissue may be a valuable indicator in determining the activity of the cells. In order to test the feasibility of O₂ consumption as a respiration indicator, we have conducted some preliminary studies in measuring O₂ consumption and CO₂ production in Dunning prostate tumor (MAT-LyLu) tissue and normal prostate tissue using Micro-Oxymax respirometer. The purpose of this study was to test whether we can use a Micro-Oxymax respirometer to detect the respiration difference between Dunning prostate tumor tissue and non-malignant tissue.

We observed that O₂ consumption and CO₂ production in 250 mg of prostate tumor tissue (Dunning R-3327; MAT-LyLu) is distinctively higher than normal rat prostate tissue of same mass in the culture system. It is very interesting to notice that during 8 hours of tissue culture, the O₂ consumption and CO₂ production by tumor tissue was more or less similar to the normal prostate tissue and beyond this point, both of these values have increased more rapidly than normal prostate tissue. The similarity in O₂ consumption and CO₂ production by prostate tumor tissue and normal prostate tissue during initial period of culture might be due to the tissue adaptation to the medium in *in vitro* system. Once they overcome this adaptation period, they behave differently in new environment. In order to determine the presence of malignant cells in the lung of tumor bearing rats, we also monitored O₂ consumption and CO₂ production in the lung tissue collected from normal and tumor bearing rats. The result showed that lung collected from tumor bearing rats following 14 days of tumor cell inoculation showed no
distinctive differences in $O_2$ consumption and $CO_2$ production from that normal lung in explant cultures. This might be due to the fact that the number of metastatic tumor cells in the lung of tumor bearing rat are not sufficient to show the effective differences in $O_2$ consumption and $CO_2$ production from that of normal lung by the Micro-Oxymax respirometer. The lung tissue collected from tumor bearing rats after 25 days of tumor cell inoculation showed a higher $O_2$ consumption and higher $CO_2$ production compared to the normal lung, indicating that Micro-Oxymax respirometer can distinguish the respiration difference between normal and malignant lung at stage when the malignant cells grew in the lungs forming small nodular structures. The above results indicate that respirometric principle might be useful in distinguishing cancer tissue from normal tissue and in evaluating the presence of malignant cells in affected organ.

The ability of growth of any cells (normal or neoplastic) is dependent upon the availability of appropriate energy metabolism to support its activities. The capability of cells to enter the process of malignancy and metastasis is related to this process. Generally normal cellular energy metabolism might be sufficient to need the energy requirement for their growth, but is insufficient for the process of growth of malignancy (Costello et al., 1994). Glucose oxidation in normal prostate epithelial cells can produce 8 ATP/mol glucose utilized via anaerobic metabolism (Costello et al., 1994). Human prostate tissues also have low respiration rate and high anaerobic glycolysis (Huggins et al., 1947). In contrast, the malignant prostate epithelial cells obtained their adenosine triphosphate (ATP) from the complete oxidation of glucose via Krebs cycle oxidation. Malignant prostate epithelial cells can generate 30 ATP/mol glucose utilized via aerobic metabolism (Costello et al., 1994). Several groups found that there are accelerated rates
of glucose transport (Flier et al., 1987; Birnbaum et al., 1987) and markedly elevated level of hexokinase in malignant liver cell (Welhouse et al., 1972). Beckner found the time course of CO$_2$ production correlated directly with motility of cancer cells (Beckner et al., 1990). Because mitochondria are the cellular organelle which involved in major metabolic process of Krebs cycle and electron transport and coupling to oxygen (O$_2$) consumption (Berner et al., 1999), damage to the mitochondria may have been responsible for the decrease in enzyme activity within it (Lin et al., 1987b). GP (±) has been shown to inhibit the sperm motility by damaging the sperm tail mitochondrial sheath observed by Electron microscope (EM) (Oko et al., 1982). Thus, the inhibitory action of O$_2$ consumption and CO$_2$ production with GP treatment in our study may involve in uncoupling of oxidative phosphorylation and inhibition of mitochondrial respiratory chain. The mitochondria’s uncoupling results in the loss of mitochondria transmembrane proton gradient and inactivation of ATPase (Benz et al., 1987).

The major task of Krebs cycle is the reduction equivalent for oxidative phosphorylation and formation of ATP. Reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH$_2$) are the major electron donors and their levels provide insight into capacity of Krebs cycle. SDH is an enzyme that transfer hydrogen molecule from succinic acid to receptor, which then catalyze an oxidation reaction to form fumaric acid in Krebs cycle. The inhibition of SDH results in the reduction of FADH$_2$ formation and subsequent ATP production. Thus, this enzyme is an essential component of Krebs cycle and a good indicator of the activity of oxidative metabolic capacity (Kondo et al., 2000). It is known that SDH inhibitor, 3-nitroprionic acid 3-np, induce a time and dose-dependent decrease of SDH activity and ATP content.
(Riepe et al., 1992). After prolonged inhibition of SDH, the activation of potassium\textsubscript{ATP}-channels (K\textsubscript{ATP}-channels) is reduced. The loss of activation of K\textsubscript{ATP}-channels is associated with the loss of mitochondrial respiration (Gross et al., 1992; Winkle et al., 1994). It is reasonable to expect that SDH activity may serve as the indicator of the degree of metabolic inhibition of cancer cells to anticancer therapy (Bemer et al., 1999) and play an important role in malignant cell metabolism (Kondo et al., 2000). Because GP (±) is lipophilic, its effects on cancer cells might be due to its lipophilic action on the cell membrane and mitochondria (Lin et al., 1991). Ferguson showed that GP (±) might reduce the succinic dehydrogenase and cytochrome oxidase activity in hen liver homogenate. He also found that SDH is susceptible to GP (±) treatment in liver homogenate of the hen (Ferguson et al., 1959). Our laboratory previously reported that GP (±) has higher binding to mitochondria than other cellular component in cultured bovine luteal cell (Lin et al., 1991). The result of present study showed that GP (±) inhibited the SDH activity of the mitochondrial fraction of MAT-LyLu cells and PC3 cells in a dose-dependent manner. Therefore, we speculate that the inhibitory effects of GP (±) on oxygen consumption and CO\textsubscript{2} production in MAT-LyLu cells and PC3 cells may be due to inhibiting the activity of mitochondrial SDH and cause a reduction in ATP production through inhibiting oxidative phosphorylation. We could not exclude the possibility of other mechanisms because of the potent enzyme inhibiting properties of GP (±).

Evidence indicates that the anticancer effect of GP (±) cannot be contributing to a single action on a selective target. Its effects might be the result of a multiple-site attack. The inhibitory effects of GP (±) on O\textsubscript{2} consumption/CO\textsubscript{2} production and SDH activity of
human prostate cancer cells provide a new evidence for understanding the mechanism of the inhibitory action of GP (±) on the growth of human prostate cancer cells. Although the exact mechanism involved in the O₂ consumption and CO₂ production remains to be elucidated, the results of present study suggest a new functional role of GP (±) on cellular oxygen phosphorylation in tumor cells. This opens the way towards understanding the mechanisms of effects of GP (±) that may be responsible for controlling prostate cancer growth. Because a high rate of proliferation is characteristic of malignant tumor cells, the clarification of energy metabolism in proliferating tumor cells is needed. The study of action of GP (±) on SDH-related energy metabolism of prostate cancer should provide important insight into the neoplastic process. Because the anticancer effects of GP (±) are associated with inhibiting SDH activity of mitochondria of prostate cancer cells, the use of selective inhibitor of prostate SDH might have a promising implication for treatment of prostate cancer patients. It could lead to improvements in therapy for prostate cancer. The information gleaned from this work might eventually help in the design of new drugs to specifically affect prostate mitochondrial respiration. This result also demonstrates the feasibility of Micro-Oxymax respirometer to detect O₂ consumption and CO₂ production in tumor cells.
Figure 3.2. Comparison of the O$_2$ consumption in normal prostate and prostate tumor (MAT-LyLu) tissue in explant culture after 14 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75x10$^6$/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as a control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 15th day following tumor cell inoculation and 250 mg of each of normal prostate tissue, and prostate tumor tissue were placed separately in a cultured flask containing 25 ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on O$_2$ consumption at different times as described in Materials and Methods section of this chapter.
Figure 3.3. Comparison of the CO₂ production in normal prostate and prostate tumor (MAT-LyLu) tissue in explant culture after 14 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75x10⁶/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as a control. Tumor bearing rats in group one and HBSS treated rat in group two were killed on 15th day following tumor cell inoculation and 250 mg of each of normal prostate tissue, and prostate tumor tissue were placed separately in a cultured flask containing 25 ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on CO₂ production at different times as described in Materials and Methods section of this chapter.
Figure 3.3
Figure 3.4. Comparison of the O\textsubscript{2} consumption by prostate cancer tissue (250 mg; MAT-LyLu), normal lung and metastatic lung in explant culture after 14 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75x10\textsuperscript{6}/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 15th day following tumor cell inoculation and 250 mg of each of prostate cancer tissue, normal lung, and metastatic lung tissue were placed separately in a cultured flask containing 25ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on O\textsubscript{2} consumption at different times as described in Materials and Methods section of this chapter.
Figure 3.4
Figure 3.5. Comparison of the CO$_2$ production by prostate cancer tissue (250 mg; MAT-LyLu), normal lung and metastatic lung in explant culture after 14 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75×10$^6$/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 15th day following tumor cell inoculation and 250 mg of each of prostate cancer tissue, normal lung, and metastatic lung tissue were placed separately in a cultured flask containing 25 ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on CO$_2$ production at different times as described in Materials and Methods section of this chapter.
Figure 3.5
Figure 3.6. Comparison of the O$_2$ consumption by prostate cancer tissue (10 mg; MAT-LyLu), normal lung and metastatic lung in explant culture after 25 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75x10$^6$/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as a control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 26th day following tumor cell inoculation and each of prostate cancer tissue (10 mg), normal lung (250 mg), and metastatic lung tissue (250 mg) were placed separately in a cultured flask containing 25 ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on O$_2$ consumption at different times as described in Materials and Methods section of this chapter.
Figure 3.7. Comparison of the CO$_2$ production by prostate cancer tissue (10 mg; MAT-LyLu), normal lung and metastatic lung in explant culture after 25 days of MAT-LyLu prostate cancer transplantation in Copenhagen rats. Viable MAT-LyLu cells (0.75x10$^6$/rat) were injected subcutaneously into the flank of rats in group one, while rats in group two were injected with equal amount of HBSS as a control. Tumor bearing rats in group one and HBSS treated rats in group two were killed on 26th day following tumor cell inoculation and each of prostate cancer tissue (10mg), normal lung (250 mg), and metastatic lung tissue (250mg) were placed separately in a cultured flask containing 25 ml RPMI-1640 medium with 5% FCS. Flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on CO$_2$ production at different times as described in Materials and Methods section of this chapter.
Figure 3.8. Effects of GP (±) on the proliferation of MAT-LyLu cells at different times after treatment (24, 28, and 32 h). The proliferation of MAT-LyLu cells with GP (±) treatment at different times was assessed by thymidine incorporation assay. MAT-LyLu cells were treated with 0.0, 1.0, and 2.0 µM of GP (±). Each bar represents the Mean ±SD of 4 wells. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.8
Figure 3.9. Effects of GP (±) on the O₂ consumption of MAT-LyLu cells. 1.5x10⁶ viable MAT-LyLu cells were cultured in 25 cm² flask that contained 5 ml RPMI-1640 medium with 5% FCS. After 24 h, the cells were treated with GP (±) at 0.0, 1.0, 2.0 μM, flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on O₂ consumption at different times after treatment (0, 24, 28, and 32 h). Each bar represents the mean ±SD of 4 flasks. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.9

![Graph showing O₂ consumption over time with different treatments and controls.](image)
Figure 3.10. Effects of GP (±) on the CO$_2$ production of MAT-LyLu cells. 1.5x10$^6$ viable MAT-LyLu cells were cultured in 25 cm$^2$ flask that contained 5 ml RPMI-1640 medium with 5% FCS. After 24 h, the cells were treated with GP (±) at 0.0, 1.0, 2.0 μM, flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on CO$_2$ production at different times after treatment (0, 24, 28, and 32 h). Each bar represents the mean ±SD of 4 flasks. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.10
Figure 3.11. Effects of GP (±) on the activity of mitochondrial succinic dehydrogenase of MAT-LyLu cells. Mitochondria were isolated from MAT-LyLu cells. Isolated mitochondria were used for succinic dehydrogenase assay. The decrease in absorbance at 600 nm was monitored for 7 min. Enzyme activity was expressed as percentage of succinic dehydrogenase activity. Each bar represents the mean ±SD of three experiments. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.14

Inhibition of Succinic Dehydrogenase (%)

Crosspool concentration (uM)

Graph showing inhibition of succinic dehydrogenase at different concentrations with error bars and significance markers."
Figure 3.12. Effects of GP (±) on the proliferation of PC3 cells at different times after treatment (24, 28, and 32 h). The proliferation of PC3 cells at different times was assessed by thymidine incorporation assay. PC3 cells were treated with 0.0, 1.0, and 2.0 μM of GP (±). Each bar represents the Mean ±SD of 4 wells. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.13. Effects of GP (±) on the O$_2$ consumption of PC3 cells. $1.5 \times 10^6$ viable PC3 cells were cultured in 25 cm$^2$ flask that contained 5 ml RPMI-1640 medium with 5% FCS. After 24 h, the cells were treated with GP (±) at 0.0, 1.0, 2.0 µM, flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on O$_2$ consumption at different times after treatment (0, 24, 28, and 32 h). Each bar represents the mean ±SD of 4 flasks. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.13
Figure 3.14. Effects of GP (±) on the CO$_2$ production of PC3 cells. 1.5x10$^6$ viable PC3 cells were cultured in 25 cm$^2$ flask that contained 5 ml RPMI-1640 medium with 5% FCS. After 24 h, the cells were treated with GP (±) at 0.0, 1.0, 2.0 μM, flasks were transferred to an incubator attached to the Micro-Oxymax respirometer. The computer automatically recorded the data on CO$_2$ production at different times after treatment (0, 24, 28, and 32 h). Each bar represents the mean ±SD of 4 flasks. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Figure 3.14
Figure 3.15. Effects of GP (±) on the activity of mitochondrial succinic dehydrogenase of PC3 cells. Mitochondria were isolated from PC3 cells. Isolated mitochondria were used for succinic dehydrogenase assay. The decrease in absorbance at 600 nm was monitored for 7 min. Enzyme activity was expressed as percentage of succinic dehydrogenase activity. Each bar represents the mean ±SD of three experiments. Bar with * represent means that are significantly different from the control group. A probability (P) of less than 0.05 was considered statistically significant.
Abstract

The role of estrogens in the prostate is unclear, but experimentally, estrogens are associated with benign prostatic hyperplasia (BPH). Estrogens were demonstrated to induce BPH in humans and dogs in the presence of androgens. For example, a time-dependent decrease in dihydrotestosterone (DHT) with an increase in the estradiol/DHT ratio in the transition zone of the aging human prostate was observed. Furthermore, in aging men, circulating androgen levels decline and the ratio of estrogens to androgens increases. The increase of estradiol/DHT ratio is considered to be responsible for a relatively estrogen-dominant environment in aging males. Dogs, like humans, exhibit the spontaneous, age-dependent occurrence of canine BPH (cBPH). This study examined effects of age and estrogenic compounds on the proliferation of specific canine prostatic cell types. We also examined keratinocyte growth factor (KGF), cyclin D1 (a cell cycle regulator) and estrogen receptor beta (ERβ) mRNA expression in these specific canine
prostatic cell types. Epithelial cells and stromal cells were prepared from the prostates of dogs at 1 and 4 years of age. Thymidine incorporation assay revealed that epithelial cells from 1-year old prostates exhibited a greater proliferation rate than the cells from 4-year old prostates. In contrast, stromal cells from 4-year old prostates proliferated more rapidly than the cells from 1-year old prostates. These results suggest that the proliferation of prostatic stromal cells increases with age and there is a greater proliferative rate in prostatic stromal cells in aging dogs. Next, in order to test whether the greater proliferative rate of prostatic stromal cells is related to the change of KGF and cyclin D1 mRNA expression in aging dogs, we examined KGF and cyclin D1 mRNA expression in specific canine prostatic cell types. We found that KGF mRNA was detected only in prostatic stromal cells from dogs of different ages, but not in epithelial cells, from dogs of different ages. In contrast to our expectations, 1–year old prostatic stromal cells possessed a higher KGF mRNA expression level than stromal cells from 4-year old prostates. Cyclin D1 mRNA was expressed ubiquitously in canine prostatic epithelial, and stromal cells, but regardless of age, higher cyclin D1 mRNA expression was observed in prostatic epithelial cells than in prostatic stromal cells. These results indicate that the changes of KGF and cyclin D1 mRNA levels in specific prostatic stromal cells are not consistent with, and do not favor, the observed age-dependent proliferative rate of prostatic stromal cells described above, and thus, do not play a critical role in the development of BPH in aging dogs. With respect to ERβ, RT-PCR results revealed that ERβ mRNA expression in both canine prostatic epithelial and stromal cells decreased with age. Higher ERβ mRNA expression was observed in prostatic epithelial cells than in prostatic stromal cells. After treatment of cultured
prostatic epithelial and stromal cells from dogs of different ages with the 17β-estradiol (E2) or zeranol (Z), a nonsteroidal anabolic agent with estrogenic activity, we found that the response of epithelial cells to E2 decreased with increasing age is consistent with the change in ERβ mRNA levels, while the response of those cells to Z treatment did not change significantly with age. In contrast, the response of prostatic stromal cells to E2 and zeranol treatment increased with age. Moreover, Treatments of E2 and zeranol did not affect the proliferation of epithelial cells from 4-year old prostate and stromal cells from 1-year old prostate. Stromal cells from 4-year old prostate responded to E2 treatment (25 nM) which increased pS2 mRNA levels of those cells, but this response was not observed in the stromal cells with zeranol treatment (25nM). It may be due to that zeranol dose is not enough to induce pS2 mRNA expression. Our preliminary result confirms our postulation. When the concentrations of zeranol is up to 50 nM, a significant induction of pS2 mRNA could be detected in the canine prostatic stromal cells. Although estrogens bind to both ERα and ERβ, they result in different response in ERα and ERβ. ERα stimulates transcription and cellular proliferation, while ERβ inactivate ERα activation. ERα and ERβ play different role in the process of cell proliferation. Estrogens stimulate the cell proliferation through ERα, while ERβ inactivates the ERα activation and decrease androgen receptor (AR) levels. Antiestrogens may play their role through ERβ. Thus, it can be speculated that the decrease in the expression of ERβ in prostatic stromal cells with increasing age results in a concomitant decrease in its negative control of ERα activation and AR levels leading to an increases in AR and ERα-mediated effects on cell proliferation and function. This can lead to the overgrowth of prostate stromal cells associated with the age-dependent
development of BPH. The results suggest that the age-dependent change in ERβ mRNA expression in the prostate may play an important role in the overgrowth of prostatic stromal cells observed in aging dogs. Zeranol increase pS2 mRNA expression level confirms its estrogenic activity. Although in our experimental condition, estrogenic potency of E2 is higher than that of zeranol, zeranol, unlike natural hormone, does not bind the carrier proteins and all may be available to the cells. Thus, zeranol may be a potential risk to humans and especially to pre-pubertal children because the endogenous E2 concentration in the body of pre-pubertal children is very low. Our results support the notion that the prostate may be susceptible to estrogenic endocrine disruption and that the overgrowth of prostatic stromal cells stimulated by estrogen and non-steroidal estrogenic compounds may be associated with the development of hBPH and cBPH. In addition, we found that KGF and cyclin D1 mRNA can be detected in adipose tissues of dogs. Thus, adipose tissues may play a role in the development of cBPH.

Introduction

The prostate is a male reproductive organ located between the bladder and urethra. In men, the prostate contributes approximately 0.5 ml of the total ejaculate volume, 2-5 ml. This portion of semen contains enzymes, proteins, electrolytes, and compounds that are thought to contribute to sperm viability. As men age, the prostate gland slowly enlarges. As this occurs, the prostate may press on the urethra and cause the flow of urine to be slower and less forceful (Wilson 1980). This condition of prostatic enlargement that is often associated with urinary obstruction in men is called benign
prostatic hyperplasia (hBPH) in men. At least 90% of men over 50 years of age are estimated to demonstrate histological evidence of prostatic hypertrophy, or hBPH. Thirty percent of these men will require surgical removal of the prostate in their lifetime (Berry et al., 1984; O’Brien et al., 1991; Osterling 1996). The current treatment options for symptomatic urinary obstructions due to hBPH include surgical resection, alpha-adrenergic blockade, hormonal manipulation (5α-reductase inhibitor and antiandrogens), transurethral resection of prostate (TURP), and microwave hyperthermia.

The growth and development of the prostate in puberty is primarily dependent on androgens, and other growth factors such as epidermal growth factor (EGF), Keratinocyte growth factor (KGF), insulin-like growth factors (IGFs)-I and –II and transforming growth factors (TGF)-α and TGF-β (Lalani et al., 1997). The prostate is also the site of conversion of testosterone (T) to dihydrotestosterone (DHT), the active metabolite of T (Isaacs and Coffey, 1979). Ninety percent of DHT are produced in stromal cells of prostate by 5α-reductase and act on the epithelial cells in a paracrine manner (Isaacs and Coffey, 1979). DHT is more potent than T and is considered an important mitogenic factor in the prostate. Evidence that the prostate gland is an androgen-dependent organ includes the demonstration that castration early in life prevents the development of both hBPH and cancer (Berthaut et al., 1997), and that the prostate gland fails to grow in patients with an inherited 5α-reductase deficiency, (Imperato-McGinley et al., 1974). However, in most men, T levels decrease with age, while estradiol levels are unchanged or increase. In elderly men, the ratio of free estradiol to free T may increase by up to 40%. Therefore, it has been speculated that estrogens may be of importance in the induction of benign prostatic hypertrophy (Vermeulen et al., 1976; Stone et al., 1986;
Ekman et al., 2000). The identification of estrogen receptor beta (ERß) (Kuiper et al., 1996) provide further evidence that estrogen may play an important role in regulating the proliferation of prostatic cells.

The androgenic regulation of the development, growth, and differentiation of canine prostate is very similar to that of the human prostate. In 85% of dogs over the age 6 years, overgrowth of the prostate, known as cBPH, is present. Indeed, since 1 year of the canine life span approximate 7 years of the human life span, the age of dogs affected by cBPH is very similar to that of humans with hBPH. This spontaneous, age-dependent occurrence of cBPH parallels that of hBPH and has rendered the hyperplastic canine prostate as an excellent animal model for the study of hBPH (Lowseth et al., 1990; Berry et al., 1986a; Berry et al., 1986b). Interestingly, in both aging men and dogs, circulating androgen levels decline (Brendler et al., 1984; Farnsworth et al., 1999; Herman et al., 2000). Thus, it can be argued that, while prostatic overgrowth in both species is certainly an age-dependent physiological condition, it is not completely an androgen-dependent event. Although it is universally accepted that castration of young and mature dogs and rats causes shrinkage of the prostate, subsequent androgen replacement to the castrated animals does not restore prostate size to pre-castration levels. This observation implies that a not-yet-defined factor(s), other than androgen, contributes to prostate growth. Banerjee and colleagues reported that Brown-Norway rats exhibited significant differences in prostatic cell death and survival among the different prostatic lobes and among rats of different ages in response to castration. The authors concluded that androgen-sensitive cell death (apoptosis) is an age-dependent and lobe-specific phenomenon in the prostate of Brown-Norway rats (Banerjee et al., 2000).
As mentioned earlier, the increase in estrogen-to-androgen ratio in aging men suggests that estrogens may play a role in the regulation of prostatic cell proliferation and possibly age-related prostatic disease. Indeed, evidence has been reported, some of which is described below, that estrogens seem to be active mainly in the stromal compartment, and may be involved in the pathogenesis of BPH. It has been shown that in the normal prostate, estrogens seem to act in a synergistic fashion with androgens in a dog model. Castrated dogs exhibited regrowth and normalized function of their prostates with exogenous androgens. When estrogens were added, the dogs developed a glandular hyperplastic prostate, including an increase in total cell number (Walsh et al., 1976). In the normal prostate gland, estrogens seem to be able to stimulate growth of the stromal compartment of the prostate. When young men given estrogen therapy, only the stromal elements of the prostate were enlarged (Schweikert et al., 1976; Krieg et al., 1981; Mobbs et al., 1990; Donnelly et al., 1983). These results demonstrated that estrogens seem to be mainly active in the stromal compartment, and are probably involved in the pathogenesis of benign prostatic hypertrophy. Krieg et al. observed that the prostatic stroma of hBPH patients compared to normal prostates contains higher concentrations of estrogen, presumably as a consequence of elevated aromatase activity (Krieg et al., 1993). In addition, age-related increases in prostate volume caused by cellular hyperplasia of basal cells of the acini and stromal cells were observed in symptomatic hBPH patients. Quantitative morphometric analysis of symptomatic hBPH patients compared to healthy controls revealed a 33% increase in stromal volume (Shapiro and Steiner, 1996). Moreover, apoptosis of prostatic stromal cells is reduced, and the lifespan of hBPH stromal cells exceeds 30 years, while epithelial cells regularly undergo programmed cell
death (apoptosis) (Claus et al., 1997). As noted earlier, hBPH is a stromal disease characterized by nodules arising in the periurethral transition zone, which is the most estrogen-responsive part of the prostate (McNeal et al., 1980). This proliferation of prostatic stroma, which obviously associated with higher plasma E2 and urinary estrogen secretion, is not correlated to testosterone (T) level (Seppelt et al., 1978). Furthermore, estrogens in the male are mainly produced by aromatization of T, a reaction that also takes place directly in the prostate stromal cells (Ekman et al., 2000), which supports the hypothesis that estrogens exert their effects on the growth and differentiation of prostate.

Estrogens have two receptors. Estrogen receptor alpha (ERα) is predominantly localized to the stromal cells of prostate, while the estrogen receptor beta (ERβ) is localized to the prostatic epithelial cells (Chang et al., 1999a). Although estrogens bind to both ERα and ERβ, these receptors mediate different responses in the target cells. ERα stimulates transcription and cellular proliferation, while ERβ diminishes ERα activation. ERα and ERβ homodimers were also shown to signal in opposite ways when complexed with Fos and Jun at an AP-1 enhancer element (AP-1). Interaction of estradiol (E2) with ERα homodimers activate transcription, whereas interaction with ERβ homodimers inhibits transcription. Moreover, the antiestrogens tamoxifen, raloxifene, and Imperial Chemical Industries 164384 (ICI164384) are potent transcriptional activators with ERβ at an API site (Paech et al., 1997). These data indicate that ERα and ERβ play different roles in the process of cell proliferation. Estrogens stimulate the cell proliferation through ERα, while ERβ diminishes the ERα activation and is a negative regulator of ERα. Although the expression of estrogen receptors in prostate is weaker than that of androgen receptors (Mobbs et al., 1990; Brolin et al., 1992), they seem to be of
importance for stem-cell growth and secretion of the extracellular matrix (Donnelly et al., 1983). Taken together, the evidence cited above, which includes elevation of the estrogen-to-androgen ratio in aging men, the experimental evidence of a role for estrogen in the regulation of prostatic cells growth, the stromal response to estrogens, the stromal nature of BPH and localization of ERβ to the stromal compartment, suggests a possible mechanism for the etiopathogenesis of hBPH.

In recent years, the occurrence of compounds possessing estrogenic activity in the environment and in food products, either as natural constituents or as contaminants, has received increasing attention because of speculation concerning their ability to adversely affect human and animal endocrinology and their possible etiological role in diseases of estrogen-sensitive tissues. The use of estrogenic anabolic growth promotant such as zeranol, has been controversial since diethylstilbestrol (DES), a synthetic estrogen that was used as an animal growth promoter, was banned by the FDA after it was demonstrated to be carcinogenic (McMartin et al., 1978). The presence of two ERs in the prostate provides an important tool for study of the roles of endogenous, as well as environmental, estrogens on prostatic function and growth. Furthermore, because estrogen-related stromal hyperplasia is seen to relate to an enhanced estrogenic status in aging men, this age-related factor is interesting with respect to its involvement in BPH induction. Until now, no study has investigated the effect of age and estrogens on the specific cell types of the prostate. In order to understand the developmental mechanism of hBPH and the effects of estrogens and a non-steroidal estrogenic compound, Z, on the proliferation of specific prostatic cell types, we investigated age-dependent changes in canine prostatic epithelial and stromal cells, as well as the effects of estrogen and Z, on
the growth of canine prostate cells from dogs of different ages.

**Method and Materials**

**Reagents**

$\alpha$-Zeranol and 17\beta-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). $\alpha$-Zeranol and 17\beta-estradiol were dissolved in 100% methanol to make 10 and 100$\mu$M stock solution respectively. The solutions were aliquoted into glass vials and stored at room temperature. Treatment solutions were prepared by the dilution of stock solution in culture medium.

**Isolation of canine prostatic epithelial and stromal cells and collection of adipose tissues**

Prostate tissue and adipose tissue samples were generous gift from Dr. Robert Hamlin (Veterinary Biosciences, The Ohio State University). Prostate tissue samples were placed into RPMI-1640 medium (GibcoBRL, Grand Island, NY) and stored at 4°C before transportation to the laboratory for cell isolation. Adipose tissue samples were collected from three different anatomical locations (peri-prostate, peri-omental, and peri-renal) of dogs and were placed into RPMI-1640 medium and stored at 4°C before transportation to the laboratory for the isolation of total RNA. The procedure for isolating canine prostatic epithelial, and stromal cells is summarized in Figure 4.1. Epithelial cells and stromal cells from canine prostatic tissues were separated as previously described by Canatan et al. (1996) with modification (canine prostatic epithelial cells were cultured in low Ca$^{2+}$ [0.04 mM] DMEM/F12; canine prostatic
stromal cells were cultured in high Ca$^{2+}$ RPMI-1640 [Ca$^{2+}$ at 0.43 mM]). Prostatic tissue (about 0.5 g) from male beagles was soaked in 70% ethanol for 30 sec, then rinsed three times in RPMI-1640 medium (GibcoBRL) containing 5% fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA). The tissues were minced (approximately 2 mm fragments) and then digested in 0.1% collagenase (GibcoBRL) in RPMI-1640 medium supplemented with 5% FCS and antibiotic-antimycotic mixture (GibcoBRL) for 18 h at 37°C. After sedimentation of the remaining undigested tissue fragments, the supernatant containing the epithelial–stromal cell mixture was centrifuged at 90 g for 5 min at 4°C to collect the cells. After resuspension of the cell pellet in RPMI-1640 supplemented with 5% FCS and antibiotic-antimycotic mixture, the cells were permitted to separate by gravity sedimentation for 20 min. The supernatant containing stromal cells and pellet containing epithelial cells, were collected into separate tubes respectively. This process was repeated 5 times with epithelial and stromal cells being pooled into respective fractions.

**Cell culture**

Canine prostatic epithelial cells were cultured in low-calcium DMEM/F12 medium (0.04 mM CaCl$_2$) (GibcoBRL) supplemented with chelex-100 (BioRad Lab., Richmond, CA)–treated 5% FCS, 10.0 μg/ml insulin (Sigma Chemical Co.), 0.02 μg/ml epidermal growth factor (GibcoBRL), 0.5 μg/ml hydrocortisone (Sigma Chemical Co.), 0.1 μg/ml cholera toxin (GibcoBRL) and antibiotic-antimycotic. The Canine prostatic epithelial cells were maintained as monolayers in four 75cm$^2$ cultured flasks at 37°C, 95% air –5% CO$_2$. Canine prostatic stromal cells were cultured in RPMI-1640 (0.43 mM
Figure 4.1 Procedure for isolating canine prostatic epithelial, and stromal cells
CaCl₂) with 5% FCS and antibiotic-antimycotic and maintained as monolayers in four 75cm² cultured flasks at 37°C, 95% air-5%CO₂. The culture medium was changed every 48h until the cells were approximately 80% confluent, at which time the cells were dissociated with 0.5% trypsin/5.3 mM EDTA in HBSS. The dissociated cells were pelleted by centrifugation at 200 xg for 5 min and then resuspended in RPMI-1640 supplemented with 5% FCS. Canine prostatic epithelial cells reached about 80% confluence (approximately 9x10⁶ cells/per 75cm² flask) after 3 days, while canine prostatic stromal cells reached about 80% confluence (approximately 6x10⁶ cells/per 75cm² flask) after 8 days Cell viability before seeding is estimated above 95% by trypan blue dye exclusion method.

**Immunocytochemical staining of primary cultured canine prostatic epithelial and stromal cells**

When canine prostatic epithelial and stromal cells reached about 80% confluence, epithelial and stromal cells were cultured in multichamber slides (Nunc Inc., Naperville, IL). After methanol fixation at −20°C for 15 min, they were processed for immunocytochemical staining. Briefly, cells were incubated with prediluted blocking serum (PBS containing 1.5% normal horse serum) for 10 min at room temperature. Cells were incubated with primary antibodies (multi-cytokeratin or vimentin mouse monoclonal antibody) (Vector Laboratories, Inc., Burlingame, CA) for 60 min at 37°C, then incubated in the presence of prediluted biotinylated universal secondary antibody (VECTASTAIN Universal Quick Kit, Vector Laboratories, Inc.) for 10 min at room temperature. They were incubated in peroxidase substrate solution [3,3’-
diaminobenzidine (DAB) peroxidase substrate kit, Vector Laboratories, Inc.] until desired stain intensity developed. Each step in the staining procedure was followed by 5 min washes in PBS.

*Thymidine incorporation assay*

Epithelial cells and stromal cells were cultured in 75 cm² flasks respectively. When 80% confluent, were harvested and seeded into 24 well culture plates (Falcon, Lincoln Park, NJ) at 2x10^4 cells and cultured for 24 h. For the measurement of basal proliferation, the cells were pulsed with 1.0 μCi/well ^3^H-thymidine (5.0 μCi/ml in cultured medium with 5% FCS). The procedure followed those described in *Materials and Methods* section of Chapter 1. For the measurement of proliferation of epithelial and stromal cells after treatment of estrogen or zeranol, the cells were treated with basal medium containing 0.2% bovine serum albumin (BSA) for 24 h and then treated with 25 nM 17β-estradiol or 25 nM zeranol for 24 h. Cells were pulsed with 1.0 μCi/well ^3^H-thymidine (5.0 μCi/ml in cultured medium with 5% FCS). The procedure followed those described in *Materials and Methods* section of Chapter 1.

*RT-PCR analysis.*

RT-PCR was used to determine the cyclin D1, KGF, and ERβ mRNA expression levels in canine prostatic epithelial and stromal cells. Total RNA from canine prostatic epithelial and stromal cells was isolated by the method described in the *Materials and Methods* section of Chapter 1. As for canine adipose tissues, total RNA was isolated by first rapidly freezing the tissues in liquid nitrogen following by homogenization with a
mortar and pestle in the presence of TRIzol Reagent (1ml/group). Subsequent steps for RNA isolation following the method described in the Materials and Methods section of Chapter 1. Reverse transcription was performed as described in the Materials and Methods section of Chapter 1. The measurement of cyclin DI mRNA was performed by PCR as described in the Materials and Methods section of Chapter 1. For the measurements of KGF, ERβ, PTPγ and 36B4 (as internal standard) mRNAs, PCR was performed by mixing 2 μl of RT product with 1.25 μl of MgCl₂ (50 mM), 2.5 μl of 10x PCR buffer, 0.2 μl of Taq polymerase (5 U/μl), and 0.3 μl of KGF, ERβ, PTPγ or 36B4 primers in a total 25 μl. For KGF, the 5’-primer was 5’-CAA TCT ACA ATT CAC AGA TAG GA-3’ and the 3’-primer was 5’-CCA TTT AGC TGA TGC ATA-3’; for 36B4, the 5’-primer was 5’-AAA CTG CTG CCT CAT ATC CG-3’ and the 3’-primer was 5’-TTG ATG ATA GAA TGG GGT ACT GAT G-3’; for ERβ, the 5’-primer was 5’-CAC CTG GGC ACC TTT CTC CTT TAG-3’ and the 3’-primer was 5’-CAG CTC TTG CGC CGG TTT TTA TC-3’; for PTPγ, the 5’-primer was 5’-GCG CAG CGA CTT CAT CCT CAC TC-3’ and the 3’-primer was 5’-GCT CGC GCT CGC CAT CCT CAC TC-3’. The PCR for KGF was run for 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min. The PCR for ERβ was run for 30 cycles of denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 1 min. The PCR for PTPγ was run for 30 cycles of denaturation at 95°C for 45 sec, annealing at 63°C for 45 sec and extension at 72°C for 1 min. The final RT-PCR products (10 μl) were separated on a 1.2% agarose gel containing ethidium bromide. The specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are presented as the ratio of KGF/36B4, cyclin
D1 /36B4, ERβ/36B4 or PTPγ/36B4. 36B4 is a cDNA clone for human acidic ribosomal phosphoprotein PO (Masiakowski et al., 1982), for which mRNA levels have been shown to be unmodified by estradiol treatment, while β-actin mRNA levels was shown to modified by estradiol (Laborda et al., 1991). Based on these observations, we select the 36B4 as the housekeeping gene for estrogen-related experiments.

The purification of the ERβ PCR products and DNA sequencing

PCR products were purified using QIAEX II kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions. Briefly, the ERβ PCR product was excised from the agarose gel, and the gel slice was weighed. Three volumes of Buffer QX1 was added to 1 volume of gel containing ERβ DNA fragment. QIAEX II was added, mixed with the Buffer QX1-gel mixture, and incubated at 50°C for 10 min. The mixture was then centrifuged for 30 sec, the supernatant carefully removed, and the pellet washed with 500 µl of Buffer QX1. After this step, the pellet was washed twice with 500 µl of Buffer PE, air-dried for 30 min, and then suspended by the addition of 20 µl 10 mM Tris-HCl (pH 8.5). DNA fragments were incubated at room temperature for 5 min and centrifuged for 30 sec. The supernatant containing purifying ERβ DNA was then collected. The purified ERβ DNA at a concentration of 10 ng/µl was sent to the Plant-Microbe Genomics Facility (PMGF) of The Ohio State University for DNA sequencing.

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) was used to
perform data 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

Morphological characteristics of primary cultured canine prostatic epithelial and stromal cells

Canine prostate epithelial and stromal cells were enriched in culture and grown to nearly 100% confluence. The canine epithelial cells were initially distinguished from stromal cells by morphological examination. Epithelial cells isolated from prostatic tissues of 1- and 4-year-old dogs grew in clusters, were polygonal in shape, and exhibited round prominent nuclei (Figures 4.2 and 4.3), while stromal cells isolated from prostatic tissues of 1- and 4-year-old dogs were characterized by a spindle-shaped appearance (Figures 4.4 and 4.5). The morphological identification of epithelial cells and stromal cells was confirmed by immunocytochemical staining for cytokeratin and vimentin, respectively. To estimate the percentage of cells staining positive for a given immunocytochemical marker, the total number of cells and the number of positive cells in three different plates were counted. The percentage of positive stained cells within a population was calculated as $100 \times \frac{\text{number of positive stained cells}}{\text{total number of cells}}$. For the cells morphologically identified as epithelial in nature, immunocytochemical staining revealed that more than 95% of these cells were positively stained for cytokeratin (Figure 4.6 A), but none stained for vimentin (Figure 4.6 B). This
finding confirmed the identity of the canine prostatic epithelial cells. In contrast, more than 95% of the stromal cells were positively stained for vimentin (Figure 4.7 A), while no staining for cytokeratin was observed (Figure 4.7 B), thereby confirming the stromal nature of this cell population. Together, these immunocytochemical findings validate the isolation and culture methods used for this study. Morphologically, stromal cells could not be distinguished based on the age of the dog from which the cells were isolated. However, the epithelial cells isolated from the 1-year-old canine prostates appeared to exhibit a greater number of binucleate cells than those from the 4-year-old canine prostate (Figure 4.2 vs. 4.3). This finding would suggest a greater rate of DNA synthesis in the epithelial cells isolated from the younger prostate. Indeed, this notion was confirmed by a quantitative assay of DNA synthesis which is described in the next section. Other features that characterize these primary cultured canine prostatic epithelial and stromal cells include the qualitative assessment of growth rate. The epithelial cells attach more rapidly and grow faster than stromal cells. 1x10^6 canine prostatic epithelial cells were cultured in low-calcium DMEM/F12 medium (0.04 mM CaCl₂), and maintained as monolayers in 75cm² cultured flasks at 37°C, 95% air-5% CO₂. The epithelial cells generally require more than one day after 80% confluence to attain 100% confluence (approximately 2x10⁷ cells/per 75cm² flask), while 1x10⁶ canine prostatic stromal cells were cultured in RPMI-1640 medium (0.43 mM CaCl₂), and maintained as monolayers in 75cm² cultured flasks at 37°C, 95% air-5% CO₂. The stromal cells need about more than two days after 80% confluence to attain 100% confluence (approximately 1x10⁷ cells/per 75cm² flask). Again, a quantitative evaluation of proliferation rates in these cell populations supports this descriptive conclusion as
Comparison of the proliferation of primary cultured canine prostatic epithelial and stromal cells from dogs of different ages.

To understand the growth characteristics of primary cultured canine prostatic cells, the cell proliferation of canine prostatic epithelial and stromal cells was assessed by the thymidine incorporation assay. Figure 4.8 shows that prostatic epithelial cells from 1-year old dogs have more DNA synthesis than those cells from 4-year old dogs. This finding suggests that epithelial cells from 1-year old canine prostates grow faster than those cells from 4-year old canine prostates. In contrast, prostatic stromal cells from 4-year old dogs exhibit more DNA synthesis than those from 1-year old dogs (Figure 4.9). The ratio of DNA synthesis of prostatic epithelial cells to prostatic stromal cells from 1-year old dogs is 2.28 (6700 DPM/well for prostatic epithelial cells vs 2940 DPM/well for prostatic stromal cells). The ratio of DNA synthesis of epithelial cells to stromal cells from 4-year dog is 0.79 (5200 DPM/well for prostatic epithelial cells vs 6560 DPM/well for prostatic stromal cells). These ratios of DNA synthesis represent the ratio of the proliferative rates between prostatic epithelial cells and stromal cells in this experiment. A high ratio (>1) indicates that the proliferative rate of prostatic epithelial cells is higher than that of prostatic stromal cells, while a low ratio (<1) means that the proliferative rate of prostatic stromal cells is higher than that of prostatic epithelial cells. It is known that hBPH is a prostatic stromal disease and symptomatic BPH patients compared to healthy controls reveal a 33% increase in stromal volume (Shapiro and Steiner., 1996). This result implies that changes in the epithelial cell/stromal cell ratio occur with
increasing age in male dogs. The observed increase in the proliferation of prostatic stromal cells in old dogs may relate to the development of cBPH.

Different expression levels of KGF mRNA in canine prostatic epithelial and stromal cells from dogs of different ages.

In the previous experiment, we found that there are differences in the DNA synthesis of specific cell types isolated from canine prostate tissues of different aged dogs (the proliferation of prostatic stromal cells increases with age). A recent publication showed that prostatic stromal cells proliferate more rapidly than epithelial cells in aging men (Claus et al., 1997). McGarvey et al. (1995) claimed that KGF, the seventh member of the FGF family (FGF-7), a potent mitogen of prostate growth, switches its paracrine loop present in normal prostate to an autocrine loop in BPH. However, it is not clear whether the expression of KGF increases with age. To determine the possible mechanism of the differences in the proliferation of prostatic specific prostatic cell types from dogs of different ages, we first examined the KGF mRNA expression in canine prostatic epithelial and stromal cells from dogs of different ages by RT-PCR. The rationale is that KGF acts as a paracrine mediator in stromal-epithelial cell interactions. KGF has been recognized as a potent mitogen secreted by prostatic stromal cells that acts through its receptor on the surface of the prostatic epithelial cells. As shown in Figure 4.10, KGF mRNA expression in stromal cells was present in dogs of different ages, but no KGF mRNA expression was detected in epithelial cells from dogs of different ages. We also observed that prostatic stromal cells from 1-year old dogs had higher KGF mRNA expression levels than those from 4-year dogs (P<0.05). The findings suggest
that young dogs produce more KGF than old dogs. This result does not support the
notion that KGF expression in prostatic stromal cells increases with age and plays a
major role in the development of cBPH. On the contrary, our findings does not indicate
that KGF exerts its effect in the aging prostate in an autocrine manner.

Different expression levels of cyclin D1 mRNA in canine prostatic epithelial and stromal
cells from dogs of different ages.

Cyclin D1 has been shown to be amplified and/or overexpressed in primary
human prostate cancer samples and several prostate cancer cell lines (Han et al., 1998).
However, the role of cyclin D1 in BPH has not been previously studied. It is not clear
whether cyclin D1 expression in specific prostatic cell types increases with age. To
determine the possible mechanism of the differences in the proliferation of specific
prostatic cell types from dogs of different ages, we examined the cyclin D1 mRNA
expression in canine prostatic epithelial and stromal cells from dogs of different ages by
RT-PCR. RT-PCR results revealed that cyclin D1 mRNA expressed ubiquitously in
canine prostatic epithelial, and stromal cells (Figure 4.11), but regardless of age, higher
cyclin D1 mRNA expression was observed in prostatic epithelial cells than in prostatic
stromal cells. These findings identified cell-specific differences in prostatic cell function,
but not age-dependent differences in cyclin D1 expression. This result indicates that
prostatic epithelial cells from dogs of different ages exhibit more cyclin D1 expression
than prostatic stromal cells, but that cyclin D1 expression in the different cell types of
dog prostate does not change with ages. Thus, this result also does not support the
hypothesis that cyclin D1 may be involved in the development of BPH in aging dogs.
Different expression of estrogen receptor beta (ERβ) mRNA in canine prostatic epithelial and stromal cells from dogs of different ages.

Estrogen, another major class of circulating steroids, was shown to be synergistically with androgens to induce cBPH in dogs by enhancing sensitivity to androgens through increasing androgen receptor levels (Moore et al., 1979). Our experimental results have shown that cultured specific canine prostatic cell types possess aromatase activity (Jiang et al., 2001). Moreover, estrogenic activity is mediated by the interaction between estrogen receptors (ERs) and the hormone with subsequent activation of the receptors. Estrogens have been speculated to exert their effects on the growth and differentiation of prostate. Estrogens have two receptors: ERα and ERβ. ERα is predominantly localized to the stromal cells of prostate, while ERβ is localized to the epithelial cells of prostate (Chang et al., 1999a). Although estrogens bind to both ERα and ERβ, ERα stimulates transcription and cellular proliferation, while ERβ diminishes ERα activation. ERα and ERβ play different roles in the process of cell proliferation. Estrogens stimulate the cell proliferation through ERα, while ERβ diminishes the ERα and is a negative regulator of ERα (Paech et al., 1997). Although ERβ mRNA has been reported to be highly expressed in the rodent and human prostate (Kuiper et al., 1996; Chang et al., 1999a), the abundance of ERα and/or ERβ in canine prostate has not been reported. It is not clear whether ERα and ERβ are present in specific prostatic cell types and whether there is differences in their mRNA expression level between specific cell types. To address these questions and to determine whether the mRNA expression of ERα and ERβ in specific canine prostatic cell types change with age, we used RT-PCR to
examine the ERα and ERβ mRNA expression in canine prostatic epithelial and stromal cells from dogs of different ages. RT-PCR detected the expression of ERβ mRNA in canine prostatic epithelial, and stromal cells from 1-year old dogs (Figure 4.12), but not in stromal cells from 4-year old dogs. Expression of ERα mRNA, however, could not be detected in our experimental samples. The primers for ERα used in this experiment were designed from human ERα sequence, rather than the canine sequence, which is slightly different from that of human ERα sequence. This may explain the negative results for this part of the experiment. Although we did not detect ERα mRNA expression in canine prostatic cells, we believe that estrogen stimulates the proliferation of prostatic cells through activating ERα. Higher ERβ mRNA expression was observed in prostatic epithelial cells from 1-year old dogs than in those cells from 4-year old dogs and stromal cells from 1-year old dogs. Regardless of age, canine prostatic epithelial cells displayed higher ERβ mRNA levels than prostatic stromal cells. These results demonstrated that ERβ expression in canine prostate exhibits cell-specific and age–dependent differences. Moreover, the results show that the ERβ mRNA levels in both epithelial cells and stromal cells decrease with age. Thus, it can be inferred that this age-dependent decrease in prostatic ERβ results in the diminished ERβ-mediated inactivation of resulting in increased ERα-mediated stimulatory effects on prostatic stromal cell function and proliferation. It an be speculated that such a process may be involved in the induction of cBPH.
Partial sequence of canine ERβ

A partial sequence of the canine ERβ cDNA is shown in Figure 4.13a. The canine ERβ cDNA was generated by RT-PCR using the primers described in the Materials and methods section of this chapter. The resultant cDNA is 355 bp in length. The canine cDNA nucleotides correspond to nucleotides 1020 through 663 of the human full-length cDNA sequence (Mosselman et al., 1996). The canine cDNA sequence shares 90.0%, 87.0%, and 86.0% nucleotide homology with the human (Figure 4.13b) (Mosselman et al., 1996), rat (Figure 4.13c) (Kuiper et al., 1996), and mouse (Figure 4.13d) (Tremblay et al., 1997) ERβ cDNAs, respectively. The canine ERβ cDNA sequence shares 84.0%, nucleotide homology with the canine ERα cDNA sequence (gi:4488119) (Figure 4.14a and 4.14b).

Effect of estrogenic compounds on the proliferation of primary cultured canine prostatic epithelial and stromal cells from dogs of different ages.

To understand the influence of estrogenic compounds on the growth of primary cultured canine prostatic cells, the cell proliferation of canine prostatic epithelial and stromal cells after treatment with estrogenic compounds was assessed by the thymidine incorporation assay. The experimental results are shown in Figure 4.15 and Figure 4.16. It appears that prostatic epithelial cells from the 1-year old dog exhibited proliferative responses to E2 treatment. Stromal cells from the same prostate however did not show positive responses to the same agent. Interestingly, when epithelial and stromal cells were isolated from a 4-year old prostate, stromal cells responded to E2 and Z, but epithelial cells did not respond to same agents (Figure 4.15 and Figure 4.16). Especially,
the results revealed that the responses of prostatic stromal cells to E2 and Z increased with age. This suggests that the sensitivity of prostatic stromal cells to estrogenic compounds increases with age, and that this elevated sensitivity may be related to the pathogenesis of prostatic stromal hyperplasia. The estrogens and zeranol may stimulate the proliferation of prostatic stromal cells through ERα activation, while they could not cause more stimulation of proliferation of prostatic stromal cells in young dogs because they activate ERβ that inactivates the ERα activation. Thus, an anti-estrogen agent specific for ERα, but not for ERβ can have potential clinical implication for the treatment of hBPH patients. Surprisingly, 4-year old stromal cells responded to zeranol. Zeranol is a nonsteroidal, anabolic growth promoter that possesses estrogenic activity. These results suggest the question of whether the long-term exposures to zeranol via the consumption of treated beef have an impact on prostate growth and function.

Effects of estrogenic compounds on expression of pS2 and PTP gamma (PTPγ) mRNA in canine prostatic stromal cells from 4-year old dogs.

pS2 is an estrogen-regulated gene and may serve as a surrogate marker of estrogen action (EL-Tanani et al., 1996; Gillesby et al., 1999). Protein tyrosine phosphatase-(- (PTPγ) is a member of the transmembrane family of protein tyrosine phosphatases that has been suggested to be involved in lung and renal cancer as a tumor suppressor gene. PTPγ is a candidate tumor suppressor gene since it is located on human chromosome 3p14.2, which is a region that is frequently deleted in renal and lung carcinomas (Wary et al, 1993; Laforgia et al., 1991). In addition, results from our laboratory have shown that E2 and Z can suppress PTPγ mRNA expression in normal and cancerous breast tissues.
(Liu et al., 2002). Thus, PTPγ may be considered as a reverse marker of estrogenic potencies. To assay and compare the estrogenic potency of E2 and Z in canine prostatic specific cells, we examined pS2 mRNA and PTPγ expression levels in prostatic stromal cells from 4-year old dogs by RT-PCR analysis. Prostatic stromal cells were treated with E2 and Z at 25 nM for 24 h. RT-PCR results showed that E2 can up-regulate pS2 mRNA expression in the stromal cells from 4-year old dogs, but Z does not induce such a change (Figure 4.17). When the concentrations of zeranol is up to 50 nM, a significant induction of pS2 mRNA could be detected in the canine prostatic stromal cells (Figure 4.18). In contrast, E2 and Z can down-regulate PTPγ mRNA expression in the stromal cells from 4-year old dogs (Figure 4.19). This findings lends further support to the finding that E2 and Z stimulate the proliferation of prostatic cells through their estrogenic activities. These results demonstrate that E2 is more estrogenically potent than Z in canine prostatic stromal cells from 4-year old dogs. A previous study has shown that estrogenic regulation of pS2 gene expression is a primary transcriptional event mediated by ER. The expression of pS2 is regulated by ER in vitro and is strongly correlated with ER in vivo (Gillesby et al., 1999). This result indicates that induction of prostatic cell proliferation by estrogenic compounds may involve a transcriptional event mediated through ERs.

Different expression of KGF mRNA and cyclin D1 mRNA in three different anatomical locations of adipose tissues from 1-year and 3-year dogs.

Adipose tissues are the site of conversion of testosterone (Schweikert et al., 1976) and production of most growth factors (Mydlo et al., 1997). Adipose stromal cells are
involved in the conversion of steroids. For example, 17β-hydroxysteroid oxidoreductase converts androstenedione to testosterone, and E1 to E2. Cytochrome P450 dependent aromatase mediates the conversion of androgens to estrogens (Ahima et al., 2000). It is known that fibroblast growth factor 2 (FGF2), a potent mitogen, for prostatic epithelial and stromal cells, were identified in human BPH tissues (Mydlo et al., 1997). Thus, we postulate that perhaps adipose tissues in addition to produce E2 may produce some growth factors, which further regulate the growth of prostate. We are interest whether the differential expression of KGF and cyclin D1 mRNA can be observed at the different locations (adjacent to prostate and distal to prostate) of adipose tissues from dogs of different ages. In order to test our hypothesis, we measured the KGF and cyclin D1 mRNA expression at three anatomical locations (peri-prostate, peri-omental, peri-renal) of adipose tissues of 1-year and 3-year old dogs. Peri-prostate adipose tissues were collected from adipose tissues adjacent to prostate gland, while peri-omental adipose tissues and peri-renal adipose tissues were collected from adipose tissues adjacent to omenta and adjacent to kidney respectively. The preliminary result shows that KGF and cyclin D1 mRNA expression can be observed at adipose tissues at three different anatomical locations (Figure 4.20 and 4.21). We also found that KGF and cyclin D1 mRNA expression levels are related to age and locations of adipose tissues. In 1-year old dog, peri-omental adipose tissues have higher KGF and cyclin D1 mRNA expression than peri-prostate and peri-renal adipose tissues. In contrast, in 3-year old dog, peri-prostate adipose tissues have higher KGF and cyclin D1 mRNA expression than the peri-omental and peri-renal adipose tissues. Furthermore, peri-prostate adipose tissues of 3-year old dog have higher KGF and cyclin D1 mRNA expression than peri-
prostate adipose tissues of 1-year old dog while peri-omental adipose tissues of 3-year old dog is lower than that of 1-year old dog. These preliminary results suggest that when dogs are young, peri-omental adipose tissues have more mitogenic activity than other two anatomical locations of adipose tissues (peri-prostate and peri-renal). When dogs are older, peri-omental adipose tissues decrease their mitogenic activity and peri-prostate adipose tissues increase their mitogenic activity. It is well known that KGF is mitogenic for the growth of prostate cells (Byrne et al., 1996). Our result implied that the KGF produced from adipose tissues may be involved in the growth of prostate in a paracrine and endocrine manner. Because the adipose tissue surrounding prostate gland can increase the production of growth factors (KGF and FGF2) and increase their influence with age, adipose tissue may be another important source of estrogen, and growth factors for prostate growth.

Discussion

The simplistic distinction that males make androgens, while females make estrogens has long been accepted. Androgens are the major sex steroid hormones that modulate the normal growth and development of the prostate. It is well known that androgens may be involve in the pathogenesis of neoplastic and hyperplastic processes of prostate (Monti et al., 1998; Bosland et al., 2000; Ho et al., 1997).

More than 60 years ago, Huggins and Hodges (Huggins et al., 1941) first introduced the concept that estrogen inhibits prostatic growth in their classic studies, in which they used the synthetic estrogen diethylstilbestrol (DES) to treat men with prostate
cancer and observed that the hormone caused a marked decrease in tumor progression. Estrogens were used to treat patients with prostate cancer mainly based on their ability to suppress androgen levels (Cox et al., 1995), but more recently, estrogens were observed to be implicated in the initiation, development, and progression of prostatic hyperplasia and carcinogenesis (Liehr et al., 2000; Miller et al., 1998; Chang et al., 1999a; Chang et al. 1999b). Fetal and neonatal exposure to estrogens results in pathological and functional changes to the prostate, and high dose T in combination with 17β-estradiol (E2) stimulates prostatic carcinogenesis in male adult rats (Vom Saal et al., 1997; Prins et al., 1992). Estrogens alone or administered together with androgens have been shown to stimulate growth in the adult and fetal prostate. Near-physiological doses of either E2 or DES given to pregnant mice have been reported to imprint on the glands of adult progeny resulting in increased prostatic weight, cell number, and androgen receptor content (Vom Saal et al., 1997). Chang and coworkers (Chang et al., 1999a; Chang et al., 1999b) have also reported that high doses of E2 given to pregnant rats can cause subsequent development of a proliferative lesion, termed dysplasia, in the prostates of progeny. There is increasing evident to support that estrogens may influence both normal and abnormal growth processes in the fetal and adult prostate (Bosland et al., 2000; Ho et al., 1997; Vom et al., 1997; Chang et al., 1999a; Chang et al., 1999b). Krieg et al found that in prostates from both normal donors (26-61 years of age) and from hBPH patient donors (50-91 years of age), the epithelial DHT level of both normal and hyperplastic glands decreased significantly with age, there was no correlation of stromal DHT levels with age in the two groups (Krieg et al., 1993). In contrast, prostatic stromal E2 and estrone (E1) levels of both normal and hBPH patients increased very significantly with age, whereas
epithelial concentrations of these estrogens were essentially equal and independent of donor age (Krieg et al., 1995). Bonnet et al (1993) and Ekman (2000) also claimed that, in the aged prostate, both normal and hBPH, there is an increase in the estrogen/androgen ratio (increase of 40% ratio) due to a decline of androgen levels. Several findings have shown that estradiol can cause a dose-dependent stimulation of prostate growth in castrated beagles (Rhodes et al., 2000). Also, administration of E2 in combination with DHT can induce cBPH in dogs (Winter et al., 1996). These findings indicate that estrogen plays an important role in the development of prostate hyperplasia.

Although many studies have established the abilities of estrogens to regulate the development of prostate hyperplasia and carcinogenesis (Liehr et al., 2000; Miller et al., 1998; Chang et al., 1999a; 1999b), no study has examined the effect of age on the specific prostatic cell types, as well as on their response to estrogen and Z. Z is an nonsteroid anabolic compounds possessing estrogenic activity that is currently approved by the FDA for use in food-producing animals. Z is used to improve feed conversion efficiency, growth rate and meat quality. Despite the FDA’s approval, concerns exist regarding the presence of Z residues in the edible tissues of implanted food animals and their impact on estrogen-responsive tissues in human consumers. Our laboratory has shown that Z, as well as the serum and extracts of edible tissues of Z-implanted cattle, possess mitogenic activity for both normal and cancerous human breast cells, and that this activity is likely to be mediated through the estrogen receptor (Lin et al., 1996). In order to determine the role of age in the development of hBPH as well as the influence of estrogens and Z on growth of specific prostatic cell types, we have measured the proliferation of specific prostatic cell types, and the mRNA expression of different genes
(KGF, cyclin D1, and ERβ) in specific prostatic cell types from dogs of different ages. We also examined the effects of estrogen and Z on the growth of these specific prostatic cell types. Reported findings have shown that ERα and ERβ play different roles in prostatic cell proliferation. ERα stimulates the cell proliferation, while ERβ restrains the stimulatory effects of ERα in rodent uterus (Weihua et al., 2000) and inhibits epithelial cell proliferation in rodent prostate (Weihua et al., 2001). This finding implies that the mRNA expression of ERα and ERβ may have different roles in the regulation of prostate cell proliferation.

Our results showed that prostatic epithelial cells from 1-year old dogs exhibit more DNA synthesis than those from 4-year old dogs. In contrast, prostatic stromal cells from 4-year old dogs exhibit more DNA synthesis than those from 1-year old dogs. Surprisingly, the ratio of DNA synthesis of epithelial cells to stromal cells from 1-year old dogs is 2.28. The ratio of DNA synthesis of epithelial cells to stromal cells from 4-year old dogs is 0.79. The ratio of DNA synthesis of prostatic epithelial cells to stromal cells present the ratio of proliferative rate between prostatic epithelial cells and stromal cells. The high ratio means that the proliferative rate of prostatic epithelial cells is higher than that of prostatic stromal cells. The low ratio means that the proliferative rate of prostatic stromal cells is higher than that of prostatic epithelial cells. It is known that hBPH is a prostatic stromal disease and symptomatic hBPH patients compared to healthy controls reveal a 33% increase in stromal volume (Shapiro and Steiner., 1996). This result suggests that changes in the epithelial cells/stromal cells ratio occur with age in male. When dogs are young, prostatic epithelial cells have more proliferation than stromal cells (ratio is high). When dogs are old, canine prostatic stromal cells have more
proliferation than epithelial cells (ratio is low). The increase in proliferation of prostatic stromal cells and decrease in prostatic epithelial cells with ages may relate to the age-related changes in the epithelial cells/stromal cells ratio in male. The decrease of this age-related ratio implied that more proliferation in the prostatic stromal cells with age may be related to the development of hBPH and cBPH. Our result is consistent with other report in which epithelial cells undergo programmed cell death (apoptosis), but no apoptotic cells can be detected in the stroma (Claus et al., 1997). In order to test whether the decrease of ratio of DNA synthesis of prostatic epithelial cells to stromal cells with age is related to KGF, cyclin D1 and ERβ expression, we first examined the KGF and cyclin D1 mRNA expression in prostatic specific cell types from dogs of different ages. This is because KGF has been reported to exhibit cell-specific mitogenic effects on the rat (Yan et al., 1992) and human prostates (Rubin et al., 1995). KGF is secreted by prostatic stromal cells that act through its receptor on the surface of the prostatic epithelial cells (Byrne et al., 1996). McGarvey et al (1995) reported that KGF mRNA expression was detected in prostatic stromal cells and basal epithelial cells from human BPH. McGarvey et al (1995) claimed that KGF could switch its paracrine loop in normal prostate to the autocrine loop in hBPH. Similarly, cyclin D1 is a very important cell cycle regulatory protein in human prostate cancer and human BPH (Han et al., 1998; Lalani et al., 1997). Cyclin D1 has been shown to be amplified and/or overexpressed in primary human prostate cancer samples and several prostate cancer cell lines (Han et al., 1998). I postulated that a greater proliferation of canine prostatic stromal cells with age might be associated with the increase in the expression of KGF and cyclin D1 in prostatic cells. However, our RT-PCR result showed that in KGF mRNA expression, the stromal cells
from 1-year old dogs showed higher KGF mRNA expression than that cells from 4-year old dogs. Moreover, no KGF mRNA expression was detected in the prostatic epithelial cells. Our result does not support the notion that the proliferation of canine prostatic stromal cells with age might be associated with role played by KGF in the cBPH in an autocrine manner. RT-PCR result does not show the more proliferation of canine prostatic stromal cells with age is related to the change in cyclin D1 mRNA expression. Thus, the more proliferation of canine prostatic stromal cells with age, associated with prostatic stromal disease, are not mediated by the change of KGF and cyclin D1 mRNA expression in aging male, but may be mediated by the increase or decrease in other gene expression.

Although estrogens have long been considered to play a minor role in male reproductive organs, the identification of a novel estrogen receptor, termed as ERβ, implied that estrogens might also play an important role in the growth of prostate. In order to test whether the proliferation of canine prostatic stromal cells with age are related to the change of ERα and ERβ mRNA expression, we examined the ERα and ERβ mRNA expression in prostatic specific cells so that we can further understand their role in the development of cBPH. Interestingly, decreased ERβ mRNA levels in both epithelial and stromal cells were observed with age. Regardless of age, ERβ mRNA expression in epithelial cells is higher than that in stromal cells. Unfortunately, we did not detect ERα mRNA expression in all prostatic cells. The possible reason is that those ERα primers were designed from human ERα sequence, and canine ERα sequence may be slightly different from human ERα sequence, those ERα primers from human ERα sequence could not detected ERα mRNA expression of canine prostatic cells. Although
we did not detect ERα mRNA expression in canine prostatic cells, we believe that estrogens exert their effect in the proliferation of prostatic cells through activating ERα. Our results demonstrated that the reduction in age-dependent ERβ mRNA expression might play important role in the development of cBPH. In the past, the direct effects of estrogens on reproductive organs, including the prostate were considered to be mediated by a single steroid hormone receptor, now referred to as estrogen receptor alpha (ERα) (Chang et al., 1999a). In 1996, however, a novel estrogen receptor subtype distinct from ERα, now termed estrogen receptor beta (ERβ), was discovered (Kuiper et al., 1996; Mosselman et al., 1996). ERα and ERβ belong to the nuclear receptor superfamily and function as ligand-activated transcription factors (Kuiper et al., 1997; Paech et al., 1997; Chang et al., 1999a). Although the precise biological function of two ER receptor subtypes in the prostate is currently undefined, it is known that ERβ, unlike ERα, is highly expressed in the rodent and human prostate (Kuiper et al., 1996; Chang et al., 1999a). Most reports showed that ERα was predominantly localized in the stromal cells of normal and hyperplastic prostate with the occasional detection of the receptor in basal cells and glandular epithelia (Kuiper et al., 1997; Chang et al., 1999; Leav et al., 2001). The expression of ERβ at both the messenger and protein levels was observed in the epithelial and stromal compartments of the normal fetal tissues and adult human prostate (Kuiper et al., 1997; Brandenberger et al., 1997; Pelletier et al., 2000; Pasquali et al., 2001; Leav et al., 2001). Estrogens were speculated to exert their effects on the growth and differentiation of prostate. Estrogens have two receptors; Estrogen receptor alpha (ERα) are predominantly localized in the stromal cells of prostate, while the estrogen receptor beta (ERβ) are localized in the epithelial cells of prostate (Chang et al., 1999a).
After estrogens bind to both ERα and ERβ, ERα stimulates transcription and cellular proliferation, while with ERβ, E2 inhibit transcription (Weihua et al., 2000). Thus, I hypothesize that ERα and ERβ may play different role in the process of cell proliferation of prostate. Estrogens stimulate the cell proliferation through ERα, while ERβ inactivates the ERα activation and inhibit abnormal growth of the prostate gland. Other findings also support my hypothesis. For example, the antiestrogens tamoxifen, raloxifene, and Imperial Chemical Industries 164384 (ICI164384) were reported to be potent transcriptional activators with ERβ at an API site (Paech et al., 1997). ERβ was demonstrated to restrain the stimulatory action of ERα in the rodent uterus (Weihua et al., 2000). ERβ activation may decrease the androgen receptor (AR) content in prostates of wild-type mice but AR level are enhanced in ERβ knockout mice (Weihua et al., 2001). ERβ knockout mice have been reported to develop age-related prostatic hyperplasia. These findings support our hypothesis that ERβ may act to inhibit abnormal growth of the prostate gland. Moreover, recent reports demonstrated that anti-estrogens down-regulate cell proliferation in human prostate cancer cells that only express the ERβ subtype (Lau et al., 2000) and a down regulation of ERβ expression occurs during prostatic carcinogenesis (Leav et al., 2001). Our results, together with other results, suggest that this change of prostatic hyperplasia may contribute to decrease in ERβ expression of the prostatic cells. The reduction of ERβ expression in prostatic cells leads to the loss of growth control process of prostate gland associated with overgrowth of prostatic stromal cells.

The role of estrogens in the prostate is unclear, but experimentally, estrogens are associated with prostatic pathology (Krege et al., 1998; Chang et al., 1999a; Chang et al., 2000).
1999b; Lau et al., 2000). In the normal prostate, estrogens seem to be able to stimulate growth of the stromal compartment of prostate. When giving estrogen therapy to young men, only the stromal elements of the prostate were enlarged (Ekman., 2000). Dogs, like humans, exhibit the spontaneous, age-dependent occurrence of benign prostatic hyperplasia. Thus, the canine prostate is an excellent model for the study of human prostatic disease. Experimentally, estrogens were demonstrated to cause a marked dose-dependent stimulation of prostate growth in the castrate dog (Rhodes et al., 2000) and induce cBPH of dogs in the presence of DHT (Winter et al., 1996). Our study demonstrated that prostatic epithelial cells from the 1-year old dog prostate responded to E2 treatment, but stromal cells from the same prostate did not show positive responses to the same agents. In contrast, stromal cells from a 4-year old prostate responded to E2 and Zeranol, but epithelial cells from the same prostate did not show positive responses to the same agents. The results indicate that the canine prostatic stromal cells from old dogs to E2 and zeranol treatment is more sensitive than the cells from young dogs. The results revealed that both cell-specific and age-dependent differences in the proliferative responses to estrogenic agents are considered to favor proliferative disorders of the prostate by altering the balance of cell growth and death between epithelial cells and stromal cells. That 4-year old stromal cells responded to zeranol implied that zeranol may be involved in the overgrowth of prostatic stromal cells in aging dogs. These results suggest that the long-term exposures to E2 and zeranol via the estrogen treatment and consumption of treated beef may have an impact on prostate growth and function. As mentioned early, hBPH was also found to be a stromal disease characterized by nodules arising in the periurethral transition zone, which is the most estrogen-responsive part of
the prostate (McNeal., 1980). The proliferation of prostatic stroma, which obviously
associated with higher plasma E2 and urinary estrogen secretion, is not correlated to
testosterone (T) level (Seppelt et al., 1978). Krieg et al also showed that estrogen
accumulation in the human prostate is an age-dependent event. The concentrations of E2
and E1 in stroma of normal and hyperplastic prostate were clearly demonstrated to
increase with age. He concluded that androgen and estrogen are responsible for the
stromal cell hyperplasia associated with hBPH (Krieg et al., 1993). Although role of
estrogens in prostate development is undefined, it is known that neonatal estronization of
rats with estradiol benzoate results in gross and histological abnormalities in the prostate
of adults including dysplasia, hyperplasia and carcinoma (Prins, 1992; Prins et al., 1993).
Significantly, fetal exposures of extremely low doses of the natural estrogen, E2, and
DES (Vom et al., 1997) or to bisphenol A (BPA) (Gupta., 2000) result in prostate
enlargement in adult mice. Gupta found that BPA and the lower dose of Des increase the
prostate enlargement associated with permanently increasing androgen receptor (AR)
binding activity of the prostate. All findings indicate that E2 and environmental
hormones through ERα activation may be involved in the overgrowth of prostatic stromal
cells, associated with BPH. Our finding suggest that the long-term consumption of low
levels of zeronol in foods derived from zeronol-implanted animal may be related to the
overgrowth of prostatic stromal cells and may have a potential risk to human health.

We next ask whether estrogen and zeronol produce the cell-specific and age-
dependent response in its target cells by binding to its receptors that regulate transcription
of estrogen-responsive genes. pS2 is a estrogen-inducible gene and can serve as a
indicator of a functional steroid-stimulatory pathway in human breast cancer cells (El-
Tanani et al., 1996). To assay and compare the estrogenic potency of E2 and zeranol in canine prostatic epithelial and stromal cells, we examine the pS2 mRNA expression in prostatic stromal cells from 4-year old dogs. We found that estrogen (E2) at 25 nM can increase the levels of pS2 mRNA expression in stromal cells from 4-year old prostate, but zeranol at 25 nM has no effects on the pS2 mRNA expression in stromal cells from same prostate. When the concentrations of zeranol increase, a significant induction of pS2 mRNA could be detected in the canine prostatic stromal cells. Although zeranol affinity for estrogen receptor is lower than E2, this result indicates that E2 is slightly higher than zeranol in induction of pS2 gene. This result is consistent with other report that pS2 gene has about equally sensitive to E2 and zeranol in human MCF-7 cells (Leffers et al., 2001). Because pS2 mRNA expression in epithelial cells and stromal cells from 1-year old prostate is too weak, we could not detect its expression in those cells. The difference between pS2 mRNA expression and cellular proliferation in stromal cells with zeranol treatment may be that the dose of zeranol is too low to induce pS2 mRNA expression. It is known that zeranol also exerts its effect partially by increasing polyamines synthesis in isolated rat uterus (Revuelta et al., 1997). It is known that polyamine levels are considered essential events for G0 to G1 transition and progression through the S phase (Chang et al., 1997). Polyamines also regulate the myc and fos in human colon carcinoma cells (Celano et al., 1989). However, we are not clear whether the proliferation effects of zeranol on prostatic cells are also partially related to polyamine synthesis. Previous study has shown that pS2 gene expression is a primary transcriptional event mediated by the ER. The expression of pS2 is regulated by ER in vitro and strongly correlated with ER in vivo (Gillesby et al., 1999). This result demonstrates that E2 is more estrogically potent
than zeranol in canine prostatic stromal cells from 4-year old dogs. Estrogenic compounds such as E2 and zeranol, may induce the cell proliferation of prostatic cells by inducing the transcriptional event and regulate the proliferation of prostate stromal cells through its receptors.

Zeranol is a nonsteroid anabolic agent with estrogenic activity. Zeranol is widely used by the meat producing industry in the United States. Human exposure to zeranol occurs though the consumption of food products, including beef and veal derived from food animals treated with zeranol. Increased pS2 mRNA expression level by zeranol confirms its estrogenic activity. Although in our experimental condition, estrogenic potency of E2 is higher than that of zeranol. Zeranol, unlike natural hormone, does not bind with carrier proteins (Mastri et al., 1985; Shrimanker et al., 1985) and all may be available to the cells. Thus, zeranol may be a potential risk to humans and especially to pre-pubertal children because the endogenous E2 concentration in the body of pre-pubertal children is very low (Klein et al., 1994). Our findings suggest that the consumption of beef or other food products derived from zeranol-treated animal may have a potential risk to human health. Preliminary data from our laboratory showed that the HPLC-detected zeranol levels in muscle tissues (meat) from zeranol-implanted beef cattle were 5.16±0.46 ng/gm. The HPLC-detected zeranol levels in muscle tissues were much lower than the permissible limits (150 ppb) of free zeranol established by FDA (Code of Federal regulation., 1991). Moreover, extracts of meat contain HPLC-detected zeranol at the levels of 0.34, 1.70 and 8.50 ng/ml, possess strong biological activity in stimulating \(^3\)H-thymidine incorporation by cultured normal human breast cells (Lin et al., 2000). Thus, the effects of the long-term consumption of low levels of zeranol in foods
derived from zeranol-implanted animal need further investigation.

The present study also demonstrated that KGF and cyclin D1 mRNA expression could be detected at three different anatomical locations of adipose tissues. Our study revealed that in 1-year old dog, the KGF and cyclin D1 mRNA expression level in peri-omental adipose tissues is relatively high compared to that of peri-prostate and peri-renal adipose tissues. While in 3-year old dog, the KGF and cyclin D1 mRNA expression level in peri-prostate adipose tissues is higher than in peri-omental and peri-renal adipose tissues. These preliminary results demonstrated that peri-omental adipose tissues have higher mitogenic activity compared to peri-prostate and peri-renal adipose tissues in young dog, while peri-prostate adipose tissues have higher mitogenic activity compared to peri-omental and peri-renal adipose tissues in older dog. It seems that peri-prostate adipose tissues increase their mitogenic activity with age and have greater influence on the development of prostate in aging dog. This result demonstrated that peri-prostate adipose tissues might play a role in the growth of prostate. It is known that adipose tissue is a source of various growth factors, sex steroids, and angiogenic substances (Ahima et al., 2000). AR, ERα and ERβ were also detected in human adipose tissues (Pedersen et al., 1996; and Pedersen et al., 2001). Our result suggests that adipose tissue may serve as a huge endocrine organ and the growth of prostate is not only regulated by prostatic KGF, but also regulated by adipose tissue KGF. Our result implies that Obese patients may have a higher risk for the hBPH. However, it is not clear whether increased dietary fat may increase the development of hBPH. Perhaps, the regulation of prostate growth is more complex than we expected.
From results of our research, we concluded that regulation of prostate function is complex. Perhaps there are many factors involved in the development of BPH and these factors interact among themselves. Estrogens and androgens cooperatively regulate the proliferation of prostatic cells. Although the mechanism of stimulatory effects of estrogens on growth of prostatic cells are still not well understood, recent research showed that estrogens may exercise a synergistic role with DHT in producing fibroblast growth factor 2 (FGF2) and KGF. FGF2 promotes an autocrine mitogenic action in the stromal cells, mediated by the FGF receptor 1 (FGF-R1), while KGF promotes paracrine mitogenic action in epithelial cells through KGF receptor (KGF-R) (a variant of FGFR2) (Byrne et al., 1996; Hermann et al., 2000). The influence of both androgens and estrogens on the promotion of smooth muscle hyperplasia seems pivotal to these complex epithelial-stromal interactions. Finding about ERβ knockout mice also indicate that ERβ is essential to develop age-related prostatic hyperplasia. Estrogens exert their effects on the growth and differentiation of prostate via ERα and ERβ. ERα is predominantly localized in the stromal cells of prostate, while the ERβ is localized in the epithelial cells of prostate. After estrogens bind to both ERα and ERβ, ERα stimulates transcription and cellular proliferation, while ERβ inhibit transcription. Estrogens stimulate the cell proliferation through ERα, while anti-estrogens down-regulate cell proliferation in human prostate cancer cells through ERβ activation. ERβ was demonstrated to restrain the stimulatory action of ERα in the rodent uterus and decreases the AR content in prostates of wild-type mice but AR level are enhanced in ERβ knockout mice. These findings support our hypothesis that ERβ may act to inhibit abnormal growth of the prostate gland. Our results show that the reduction of ERβ expression in prostatic cells
with age results in enhanced AR levels and more ERα activation, which lead to the overgrowth of prostatic stromal cells. ERβ is now thought to be an important regulator to modulate the growth and development of prostate. The decrease in control of growth process of prostate mediated by ERβ with age may play important role in the formation of prostatic stromal hyperplasia. It is possible that antiestrogens will come to play a role in the treatment of benign prostatic hypertrophy. Thus, the development of new estrogen antagonist, specifically binding for ERβ, may be our strategy against human prostatic hyperplasia (BPH) in future. The regulation of prostate growth is more complex than we expected. The growth of prostate is not only regulated by prostatic KGF, but also regulated by adipose tissue KGF. Adipose tissues may be a huge endocrine organ.
Figure 4.2. Phase contrast photomicrograph of cultured canine prostatic epithelial cells from 1-year dog. Canine prostatic epithelial cells from prostate specimens of 1-year dog were isolated as described in the Materials and Method section. Normal canine prostatic epithelial cells were cultured in low Calcium DMEM/F12 medium (0.04 mM CaCl₂) with 5% FCS for more than one day after 80% confluence. Epithelial cells grew in clusters, were polygonal in shape, and have round prominent nuclei. 1-year-old epithelial cells contained a higher number of binucleate cells than 4-year-old epithelial cells (400x magnification).
Figure 4.2
Figure 4.4
Figure 4.4. Phase contrast photomicrograph of cultured canine prostatic stromal cells from 1-year dog. Canine prostatic stromal cells from prostate specimens of 1-year dog were isolated as described in the Materials and Method section. Normal canine prostatic stromal cells were cultured in RPMI-1640 medium (0.43 mM CaCl₂) with 5% FCS for more than two days after 80% confluence. Stromal cells grew slower in culture than epithelial cells and were characterized by a spindle-shaped appearance (400x magnification).
Figure 4.4
Figure 4.5. Phase contrast photomicrograph of cultured canine prostatic stromal cells from 4-year dog. Canine prostatic stromal cells from prostate specimens of 4-year dog were isolated as described in the Materials and Method section. Normal canine prostatic stromal cells were cultured in RPMI-1640 medium (0.43 mM CaCl₂) with 5% FCS for more than two days after 80% confluence. Stromal cells grew slower in culture than epithelial cells and were characterized by a spindle-shaped appearance (400x magnification).
Figure 4.6. Immunocytochemistry of cultured canine prostatic epithelial cells from canine prostate tissues. Canine prostatic epithelial cells from canine prostate specimens were stained as described in the Materials and Method section. Greater than 95% of the epithelial cells were positively stained for cytokeratin (A), but were negative for vimentin (B) (100x).
Figure 4.6
Figure 4.7. Immunocytochemistry of cultured canine prostatic stromal cells from canine prostate tissues. Canine prostatic stromal cells from canine prostate specimens were stained as described in the Materials and Method section. Greater than 95% of the stromal cells were positively stained for vimentin (A), but were negative for cytokeratin (B) (100x).
Figure 4.7
Figure 4.8. Comparison of proliferation of canine prostatic epithelial cells isolated from dogs of different ages. The proliferation of canine prostatic epithelial cells isolated from prostate tissues of 1-year and 4-year dog respectively. Canine prostatic epithelial cells were cultured in 24 well plate for 24 h. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 4.8

N=4
Mean±SD
*P<0.05

Thymidine incorporation (1x10^3)

Age (years)
Figure 4.9. Comparison of proliferation of canine prostatic stromal cells isolated from dogs of different ages. The proliferation of canine prostatic stromal cells isolated from prostate tissues of 1-year and 4-year dog respectively. Canine prostatic stromal cells were cultured in 24 well plate for 24 h. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 4.9

Thymidine Incorporation (1x10^3 DPM/well)

N=4
Mean±SD
*P<0.05

Age (years)

1 4
Figure 4.10. Expression of KGF mRNA expression in canine prostatic epithelial and stromal cells from dogs of different ages. Canine epithelial, and stromal cells were isolated from the prostate tissues of 1-year and 4-year dogs. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. KGF mRNA expression was detected in canine prostatic epithelial and stromal cells by RT-PCR analysis. Each bar represents the Mean ±SD of 3 experiments. Bar with * represent 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 4.10

Epithelial cells

Stromal cells

Relative expression ratio (KGF/36B4)

1 year

4 year

*P<0.05

N=3

Mean±SD

36B4 (563 bp)

KGF (318 bp)
Figure 4.11. Expression of cyclin D1 mRNA expression in canine prostatic epithelial and stromal cells from dogs of different ages. Canine epithelial, and stromal cells were isolated from the prostate tissues of 1-year and 4-year dogs. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. Cyclin D1 mRNA expression was detected in canine prostatic epithelial and stromal cells by RT-PCR analysis. Each bar represents the Mean ±SD of 3 experiments.
Figure 4.11
Figure 4.12. Expression of estrogen receptor β (ERβ) mRNA expression in canine prostatic epithelial and stromal cells from dogs of different ages. Canine epithelial, and stromal cells were isolated from the prostate tissues of 1-year and 4-year dogs. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. ERβ mRNA expression was detected in canine prostatic epithelial and stromal cells by RT-PCR analysis. Each bar represents the Mean ±SD of 3 experiments. Bar with * represent 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 4.12
Figure 4.13. Partial sequence of canine estrogen receptor β (ERβ). Sequence analysis of the canine ERβ cDNA is 355 nucleotides in length (Figure 4.13a). The sequence shares 91.0%, 87.0%, and 86.0% homology with the human (Figure 4.13b), rat (Figure 4.13c), and mouse (Figure 4.13d) cDNAs, respectively. The nucleotide sequence corresponds to the 3’ untranslated region of the human ERβ cDNA. Bolded nucleotides are different among species.
Figure 4.13a
Figure 4.13b
Rattus norvegicus estrogen receptor 2 (Esr2), mRNA
Length = 2555
Score = 333 bits (168), Expect = 9e-89
Identities = 311/355 (87%), Gaps = 3/355 (0%)
Strand = Plus / Minus

Query: 1 cagctctttgcgccggttttatcttttgctatgtaatca 60
Sbjct: 883 cagcttttacgccggttcttgtctatggtacactgattcgtggctggacagatataatca 824

Query: 61 ttatgtcctttgaatgcttcttttaaaaaaggccttacatccttcacacgaccagactcca 120
Sbjct: 823 ttatgtcctttgaatgcttcttttaaaaaaggccttacatccttcacatgaccaaacgccg 764

Query: 121 tagtgatatccagatgcgtaatcgctgcagaccgcacagaagtgggcatccctctttgaa 180
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Query: 181 cttggactagtaatggggctggcacaactgctcccactaagcttcctcttcagtgtctct 238
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Query: 239 ctgctttacaggttaagg-gggttctcgtatcttgctctcaccaccaagctcctttttaggt 297
Sbjct: 643 ctgctttacaggttaagg-gggttctcgtatcttgctctcaccaccaagctcctttttaggt 584

Query: 298 ttgcatacaagaagtgacaacttggaatgatgctaaaggnnaaaggtgcccag 352
Sbjct: 583 ttgcatagagggagcatgatgattggcaatggtgctgaagagaagctgcccag 529

Figure 4.13c
Mus musculus estrogen receptor 2 (beta) (Esr2), mRNA

Length = 2152
Score = 303 bits (153), Expect = 8e-80
Identities = 306/355 (86%), Gaps = 3/355 (0%)
Strand = Plus / Minus

Query: 1 cagctctttgacccggtttttatctttgtactgatgtatctggtcatatactca 60

Sbjct: 478 cagcttttaagcccggtttttatctttgtactgatgtatctggtcatatactca 419

Query: 61 ttatgtctttgaatgctttcttttaaaaaaggccttacatccttcacacgaccagactcca 120

Sbjct: 418 ttatgtctttgaatgctttcttttaaaaaaggccttacatccttcacacgaccagactcca 359

Query: 121 tattgatattccagatgcgatctgctgcagaccgcacagaagtgggcatccctctttgaa 180

Sbjct: 358 tattgatattccagatgcgatctgctgcagaccgcacagaagtgggcatccctctttgaa 299

Query: 181 cttggacctgtaatgggtcggcaacagctttttttcttcgttaacttaacctgccccc 238

Sbjct: 298 cttggacctgtaatgggtcggcaacagctttttttcttcgttaacttaacctgccccc 239

Query: 239 ctgcttacaggttaagg-gggttcagttcagttcttcacaccaaggtctttttgggt 297

Sbjct: 238 ctgcttacaggttaagg-gggttcagttcagttcttcacaccaaggtctttttgggt 179

Query: 298 tctgcatacaagtgcaacatggcaatggcagttacaaaaggtctggcccaag 352

Sbjct: 178 tctgcatacaagtgcaacatggcaatggcagttacaaaaggtctggcccaag 124

Figure 4.13d
Figure 4.14. Compare the sequence of canine estrogen receptor β (ERβ) cDNA with the sequence of canine estrogen receptor α (ERα) cDNA. Sequence analysis of the canine ERβ cDNA reveals that the sequence of canine ERβ cDNA shares 84.0% homology with the sequence of canine ERα cDNA (gi:14488119) (Figure 4.14a and 4.14b). The nucleotide sequence corresponds to the 3’ untranslated region of the canine ERβ cDNA. Bolded nucleotides are different between canine ERβ and ERα cDNAs.
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<td>CTGGAAGAAGC</td>
<td>GCCTTACAGC</td>
<td>CTTCACAGGA</td>
<td>CCAGACCTCA</td>
</tr>
</tbody>
</table>

**Figure 4.14a**
Canis familiaris partial mRNA for ERalpha protein (eralpha gene)
Length = 748

Score = 91.7 bits (46), Expect = 6e-16
Identities = 103/122 (84%)
Strand = Plus / Minus

Query: 1 cagctctttgcccgtttttatctttgtaacgcttttagtctctttgtatctatgcgctttgatagtaatca 60

Sbjct: 414 cagctctttgcccgtttttatctttgtaacgcttttagtctctttgtatctatgcgctttgatagtaatca 355

Query: 61 ttatgctctttgcccgtttttatctttgtaacgcttttagtctctttgtatctatgcgctttgatagtaatca 120

Sbjct: 354 ttatgctctttgcccgtttttatctttgtaacgcttttagtctctttgtatctatgcgctttgatagtaatca 295

Query: 121 ta 122

Sbjct: 294 ta 293

Figure 4.14b
Figure 4.15. Effects of estrogenic compounds on the proliferation of cultured prostatic epithelial cells from dog of different ages. Canine epithelial cells were isolated from the prostate tissues of 1-year and 4-year dogs. The proliferation of epithelial cells was assessed by thymidine incorporation assay. Epithelial cells were treated with 17β-estradiol (E2) and zeranol 25 nM for 24 h respectively. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 4.15

Thymidine incorporated (1×10^6)

Control  E2  Z

1 year EC  4 year EC

N=4 (well)
Mean±SD
*P<0.05
Figure 4.16. Effects of estrogenic compounds on the proliferation of canine prostatic stromal cells from dog of different ages. Canine prostatic stromal cells were isolated from the prostate tissues of 1-year and 4-year dogs. The proliferation of stromal cells was assessed by thymidine incorporation assay. Stromal cells were treated with 17β-estradiol (E2) and zeranol 25 nM for 24 h respectively. Each bar represents the Mean ±SD of 4 wells. Bar with * represent 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 4.16
Figure 4.17. Effects of estrogenic compounds (25 nM) on the pS2 mRNA expression of cultured canine prostatic stromal cells from 4-year dog. Canine stromal cells were isolated from the prostate tissues of 4-year dogs. Stromal cells were treated with 17β-estradiol (E2) and zeranol 25 nM for 24 h respectively. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. pS2 mRNA expression was detected in canine prostatic stromal cells by RT-PCR analysis. Each bar represents the Mean ±SD of 3 experiments. Bar with * represent 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 4.17
Figure 4.18. Effects of estrogenic compounds (50 nM) on the pS2 mRNA expression of cultured canine prostatic stromal cells from 4-year dog. Canine stromal cells were isolated from the prostate tissues of 4-year dogs. Stromal cells were treated with 17β-estradiol (E2) and zeranol 50 nM for 24 h respectively. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. pS2 mRNA expression was detected in canine prostatic stromal cells by RT-PCR analysis.
Figure 4.18

Relative expression ratio
(pS2/36B4)

0.0 0.1 0.2 0.3 0.4 0.5

Control

Z

E2
Figure 4.19. Effects of estrogentic compounds on the PTP gamma (PTP\(\gamma\)) mRNA expression of cultured canine prostatic stromal cells from 4-year dog. Canine stromal cells were isolated from the prostate tissues of 4-year dogs. Stromal cells were treated with 17\(\beta\)-estradiol (E2) and zeranol 25 nM for 24 h respectively. Total RNA was isolated from these cells and RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. PTP\(\gamma\) mRNA expression was detected in canine prostatic stromal cells by RT-PCR analysis.
Figure 4.19

Relative expression ratio

pTP gamma/36B4

< 1
Figure 4.20. Expression of KGF mRNA expression in canine adipose tissues from 1-year and 3-year dogs at different anatomical locations. Canine adipose tissues at different locations were obtained from 1-year and 3-year dogs. Total RNA was isolated from these cells as described in Materials and methods section of Chapter 4. RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. KGF mRNA expression in canine adipose tissues at three different anatomical locations was detected by RT-PCR analysis.
Figure 4.20
Figure 4.21. Expression of cyclin D1 mRNA expression in canine adipose tissues at different anatomical locations. Canine adipose tissues at different locations were obtained from 1-year, and 3-year dogs. Total RNA was isolated from these cells as described in Materials and methods section of Chapter 4. RT-PCR analysis was performed as described in Materials and methods section of Chapter 1. Cyclin D1 mRNA expression in canine adipose tissues at three different anatomical locations was detected by RT-PCR analysis.
Figure 4.21
Gossypol [GP (±)] is a naturally occurring yellow pigment present in cottonseed products. GP (±) has been shown to possess the antiproliferative properties in prostatic cancer cells. Since the mechanism of GP’s action is not defined, we utilized in vitro cell culture systems to evaluate the effects of GP (±) on prostate cancer cell lines. The result showed that GP (±) significantly inhibited the proliferation of three prostate cell lines (PC3, MAT-LyLu, and LNCaP) in a dose-dependent manner. Among the three cell lines tested, LNCaP cells appeared to be the least sensitive to the inhibitory effect of GP (±). Cultured MAT-LyLu cells however were 4 fold and 16 fold more sensitive to the proliferation of to GP (±) than PC3 and LNCaP cells, respectively. While a concentration of 0.25 μM GP (±) was sufficient to achieve significant growth inhibition in MAT-LyLu cells, PC3 and LNCaP cells required 1.0 μM and 4.0 μM, respectively to achieve a similar levels of growth inhibition. Furthermore, among the androgen-independent prostate cancer cells tested in this study, the faster growth rate was associated with a greater sensitivity to GP (±) treatment. For example, the proliferation rate
of MAT-LyLu cells was about 8.1-fold higher than that of PC3 cells (94x10^3 DPM/well vs 11.6x10^3 DPM/well in DNA synthesis). Similarly, the DT of MAT-LyLu cells is shorter than that of PC3 cells (8.9 h vs 22.48 h). These indicators of faster growth rate of MAT-LyLu cells are associated with the sensitivity of MAT-LyLu cells to the antiproliferative action of GP (±) which is approximately 4-fold higher than that of PC3 cells. Interestingly, both GP (-) enantiomers, and refined cottonseed oil possessed antiproliferative effects on PC3 cells. PC3 cell proliferation was significantly suppressed by treatment with GP (-) at 1.0 μM, and 5% refined cottonseed oil. The GP (-) and refined cottonseed oil exhibited inhibitory doses that represents a greater potency than that of GP (±). Since transforming growth factor-β (TGFβ) is a well-known negative regulator of prostate growth and in light of our previously published findings (Shidaifat et al., 1996; Shidaifat et al., 1997). We speculated that TGFβ may be involved in mediating the effects of GP (±). RT-PCR and ELISA results showed that GP (±) induces TGFβ1 mRNA expression and TGFβ1 protein production in PC3 and MAT-LyLu cells. Similarly, refined cottonseed oil also elevated TGFβ1 mRNA expression in PC3 cells. GP (±), GP (-) and refined cottonseed oil were also shown to reduce cyclin D1 mRNA expression in PC3 cells. GP (±) induced a similar effect in LNCaP cells, but at a higher dose (8.0 μM). These results indicate that the inhibitory effects of GP (±) and refined cottonseed oil on cellular proliferation of human prostate cancer may be associated with induction of TGFβ1, which in turn influences the expression of cell cycle regulatory protein cyclin D1 that regulate cell cycle progression of prostate cancer cells. Based on the results of the effects of GP (±) on LNCaP cells, it is evident that TGFβ1 mediate only a portion of GP’s
antiproliferative effects and that perhaps there are other mechanisms through which GP (±) can inhibit prostate cell growth.

The MLL cell is a novel subline of the MAT-LyLu cell line isolated from metastasized lungs of MAT-LyLu-bearing Copenhagen rats. We compared the MLL cells to the parental MAT-LyLu cells with respect to invasive ability, mRNA expression level for nm23 metastasis suppressor gene, and response to GP (±). MLL cells were more invasive than MAT-LyLu cell. The nm23 gene has been used as a measure of metastatic potential. Consistent with the invasiveness findings, MLL cells possessed lower steady state levels of nm23 mRNA than MAT-LyLu cells. In addition, both MLL and MAT-LyLu cells were susceptible to GP (±), which induced a dose-dependent inhibition of invasive activity with a concomitant increase in the mRNA expression of nm23 metastasis suppressor gene. These results imply that the inhibitory actions of GP (±) on MLL and MAT-LyLu cells is mediated by elevating the expression of nm23 metastasis suppressor gene.

The proliferation of tumor cells depends on the supply of nutrients and O₂. Since GP (±) has been shown to have antimitochondrial properties and to depress oxygen uptake in the guinea pig keratinocyte, it may be an effective agent to inhibit the oxidative phosphorylation of tumor cells. We found that GP (±) significantly inhibited the O₂ consumption and CO₂ production of cultured MAT-LyLu and PC3 cells in association with reductions in DNA synthesis and in the activity of mitochondrial succinic dehydrogenase in those cells. These results suggest that the inhibitory effect of GP (±) on the O₂ consumption and CO₂ production of prostatic tumor cells may relate to the
disruption of oxidative phosphorylation by inhibiting mitochondrial succinic dehydrogenase activity in those cells.

The role of estrogen in the prostate is unclear, but experimentally, estrogens are associated with benign prostatic hyperplasia (BPH). An increase in the proliferation rate of canine prostatic stromal cells with age was observed. Canine prostatic stromal cells from dogs of different ages exhibited cell-specific differences in aromatase activities. Prostatic stromal cells from 1-year and 3-year old dogs were approximately 2.16 and 10.79-fold higher, respectively, than that in age-matched epithelial cells (Jiang et al., 2001). This result demonstrates that the conversion of testosterone to estrogen in canine prostatic stromal cells is higher than in canine prostatic epithelial cells. This result indirectly indicates that canine prostatic stromal cells exhibit greater estrogen synthesis than canine prostatic epithelial cells. A decrease in ERβ mRNA expression in canine prostatic cells with age was observed. It is known that estrogens stimulate cell proliferation through ERα, while ERβ counters the ERα activation and inhibits abnormal growth of the prostate gland. Recent reports demonstrated that anti-estrogens downregulate cell proliferation in human prostate cancer cells through ERβ. Thus, ERβ may be considered to be a negative regulator of growth of the prostate gland. Our results suggest that a decrease in ERβ expression may contribute to prostatic hyperplasia. The reduction of ERβ expression in prostatic cells results in the greater ERα activation and enhanced AR levels which lead to the overgrowth of prostatic stromal cells. Such a decrease in the control of prostate gland by ERβ may be associated with the development of canine BPH.

The response of canine prostatic epithelial cells to estradiol (E2) treatment decreased with age, while that of prostatic stromal cells to E2 and zeranol treatment increased with age.
We found that estrogen (E2) at 25 nM can increase the levels of pS2 mRNA expression in stromal cells from the same prostate, but zeranol at 25 nM had no effect on the pS2 mRNA expression in stromal cells from same prostate. When the treatment concentrations of zeranol were increased, a significant induction of pS2 mRNA could be detected in the canine prostatic stromal cells. This result suggests that the response of canine prostatic cell types to E2 treatment is through ERα and is cell-specific and age-dependent. This also implies that environmental hormones may pose a potential risk to human health.

In summary, GP (±) is a potent chemopreventive and chemotherapeutic agent against prostate cancer. The anti-cancer ability of GP (±) to induce TGFβ1, induce nm23, inhibit 5α-reductase, and interfere with mitochondrial succinic dehydrogenase in prostate suggests that it has a great potential to become an antimetastatic and antitumor agent. In addition, the growth inhibitory effects of dietary cottonseed oil against prostate cancer also provides another avenue for the development of clinical applications for use of GP (±) or GP-containing foods. The stimulation by E2 and zeranol to canine prostatic cell types is cell-specific and age-dependent, suggesting that estrogens and androgens cooperatively regulate the proliferation of prostatic cells. The decrease in ERβ mRNA expression in prostatic cells with age causes a greater ERα activation and enhanced AR levels which lead to the overgrowth of prostatic stromal cells, which is associated with the development of BPH.


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