Indole-3-carbinol and 3’, 3’-diindolylmethane modulate androgen's effect on C-C chemokine ligand 2 and monocyte attraction to prostate cancer cells

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Abstract

Inflammation has a role in prostate tumorigenesis. Recruitment of inflammatory monocytes to the tumor site is mediated by C-C chemokine ligand 2 (CCL2) through binding to its receptor CCR2. We hypothesized that androgen could modulate CCL2 expression in hormone-responsive prostate cancer cells and thereby promote recruitment of monocytes. Given the inhibitory effect of broccoli-derived compounds indole-3-carbinol (I3C) and 3,3’-diindolylmethane (DIM) on androgen-dependent pathways, we also reasoned that I3C and DIM could modulate the effect of androgen on CCL2-mediated pathways.

Dihydrotestosterone (DHT) was found to induce a time (0-72 hrs) and concentration-dependent (0-1 nM) increase in CCL2 mRNA levels in androgen-responsive human prostate cancer cells (LNCaP). This increase in CCL2 mRNA corresponded with increased secretion of CCL2 protein. The effect of DHT was mediated through an androgen receptor (AR)-dependent pathway as small inhibitor RNA against AR negated the induction of CCL2. Although DHT also induced TWIST1 mRNA, an epithelial-mesenchymal transition related factor and purported inducer of CCL2, blocking its expression with small inhibitor RNA did not inhibit DHT induction of CCL2 mRNA. Moreover, conditioned media from androgen-treated cells promoted human monocyte THP-1 cell migration and this effect was blocked by antibody against CCL-2.

Both I3C and DIM inhibited promotional effects of DHT on CCL2 and migration. These results demonstrate that androgen may regulates CCL2 and promotes inflammatory micro-environments in prostate tumors, and that this process can be blocked by broccoli-derived compounds.
Introduction

Androgens are functionally required for the normal growth and development of the prostate gland. In adult males, androgens promote secretary epithelial cell survival. However, androgens also promote prostate tumor development and progression (1,2). Androgen deprivation is the only clinically effective therapy for advanced prostate cancer. However, because of the relapse of castration-resistant androgen independent tumors, the long-term benefit of androgen deprivation in patients with metastatic disease remains controversial (3,4). Although previous research shows many of androgens biological effect are likely through regulation of androgen responsive genes (ARG) via an androgen receptor-mediated pathway (5), some of the molecular effects of androgens in normal and prostate cancer remain unresolved.

Inflammation as a causal agent has been linked to approximately 20% of human cancers (6). In prostate cancer, a growing amount of evidence suggests a link between prostate inflammation and subsequent cancer development (7-12). Previous studies suggest C-C chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1) may play pivotal role in prostate cancer tumorigenesis and invasion (13,14). CCL2 is known to attract monocytes to the site of inflammation, and by binding to its receptor CCR2, directly stimulates prostate cancer cell proliferation, survival, and migration (15). Prostate cancer cells LNCaP, C4-2B, PC-3, and VCaP produce CCL2 (16,17). Furthermore, recent findings suggest a role for CCL2 in acquisition of epithelial-mesenchymal transition (EMT) properties (18). EMT has been shown to be crucial for the pathogenesis of tissue fibrosis and cancer (19). Prostate tumor epithelial cells gain the ability to migrate and invade by differentiating through activation of biological pathways associated with EMT. Androgens were reported to induce changes that are characteristic of EMT such as expression of TWIST-1, CDH1 and SNAIL, cytoskeleton reorganization, and promotion of metastatic behavior of castration-resistant prostate cancer cells (20). TWIST-1, a helix-loop-helix transcription factor thought to be an important mediator of EMT, is up-regulated in several types of epithelial cancers including prostate, breast and gastric carcinomas (21-24). Several EMT-related genes such as CDH1, CDH2, SNAIL, desmin, vimentin, are putative down-stream targets regulated by TWIST-1. TWIST-1 also appears to be involved in inflammatory pathways (25). TWIST-1 expression in T helper 1 (Th1)
lymphocytes and bone marrow-derived macrophages attenuates the expression of interferon-γ, IL-2, and TNF-α, further implicating TWIST-1 in the regulation of cytokine expression (25-27). It was also reported that CCL2 expression may be subject to regulation by TWIST-1 (28). The relationship between androgens and these EMT-related proteins, as well as the relation between TWIST-1 and CCL2 in prostate cells remain unresolved.

Cancer prevention represents an integral part of a sound strategy in fight against cancer. Diet-derived compounds are often the focus due to their perceived safety, Indole-3-carbinol (I3C) is a putative preventive compound derived from hydrolysis of glucobrassicin from ingesting cruciferous vegetables such as broccoli, cabbage and cauliflower (29). I3C can be converted to the dimeric form 3’,3’-diindolylmethane (DIM) in stomach (30,31). Previous studies, including our own, have shown that the I3C and DIM inhibit prostate cancer cell growth in vitro and in vivo (32-34). Both I3C and DIM appeared to exert their effects in part through modulation of androgen-dependent pathways. In addition, these agents have also been reported to prevent or delay the progression of cancer, through their ability to attack cancer stem cells or EMT-type cells and modulate inflammation in cancer cells (35,36). The effects of these compounds on prostate cancer-related EMT remains unknown.

Given the potential roles of androgen in regulating prostate cancer and EMT and existent information on TWIST-1 and CCL-2, we hypothesized that androgen may modulates TWIST-1 and in turn CCL2 expression in prostate cancer cells, and these effect may resulted in promotion of monocytes recruitment. The present study seeks to resolve these interactions. Finally, given the effects of I3C and DIM on androgen-dependent pathways (32-34), we also sought to determine the effects of I3C and DIM on TWIST-1, CCL2 and monocyte migration.
Materials and Methods

Chemicals and Reagents. I3C, DIM, dihydrotestosterone (DHT), and DMSO were from Sigma Chemical (St. Louis, MO). Trizol, AffinityScript Multiple Temperature cDNA Synthesis kit and Taqman real-time PCR primers and probes were obtained from Life Technology (Grand Island, NY). On-Target plus SMART pool siRNA targeting androgen receptor (AR) and TWIST-1 were purchased from Thermo Fisher Scientific (Waltham, MA). HiPerFect Transfection Reagent was purchased from Qiagen (Valencia, CA, USA).

Cell culture. LNCaP and PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Media A (RPMI 1640 medium with phenol red and 2mM L-glutamine (Invitrogen/Life Technologies, Grand Island, NY), 100 U/mL penicillin and 100 mg/mL streptomycin (BioSource International, Camarillo, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies). For androgen-related experiments, cells were seeded as follows: 6-well plates at 250,000 cells/well. After 24 h in medium A, cells were switched to medium B, which had the same composition as Media A except that FBS was replaced with 10% charcoal dextran-treated FBS (GIBCO/Life Technologies) for an additional 24 h to minimize background androgen levels. Cells were incubated in the presence of 5% CO₂ in air at 37°C. THP-1 cell were also purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium with phenol red and 2mM L-glutamine (Invitrogen/Life Technologies, Grand Island, NY), 100 U/mL penicillin and 100 mg/mL streptomycin (BioSource International, Camarillo, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies)

RNA Isolation and Real-Time Reverse Transcriptase PCR. Total RNA was isolated using TRIzol reagent (Invitrogen/Life Technologies, Grand Island, NY), and reverse transcribed to cDNA using AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent/Life Technologies). Real-time PCR was carried out using a TaqMan Fast Universal PCR Master Mix on a 7900HT FAST real-time PCR System (Applied Biosystems/Life Technologies). The amplification parameters used were as follows: 95°C for 20 s, followed by 46 cycles of amplification at 95°C for 1 s and 60°C for 20 s. Relative mRNA fold changes
to control were calculated using the comparative CT (2−ΔΔCt) cycle (ΔCt) method following manufacturer's directions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for all gene expression analysis calculation.

Determination of gene expression in LNCaP cells using RT-PCR. To examine the effects of test compounds on TWIST1, CDH1, CDH2, SNAI1 (SNAIL1), DES (desmin), VIM (vimentin), KLK3 (PSA), CCL2 or TNF (TNF-α) mRNA levels, LNCaP cells were plated in six-well plates (0.25×10^6 cells/well) in Media A and after 24 h the media was removed and replaced with fresh media containing vehicle or varied concentrations of test compounds I3C at 5, 25, 50 µM and DIM at 1, 5, 10 µM. LNCaP cells were plated in six-well plates (0.25×10^6 cells/well) in Media A, and switched to Media B 24 h after plating to minimize the effect of serum hormones. The cells were then incubated in Media B for an additional 24 h before the treatments began. Twenty-four hours later, the medium was replaced with fresh medium containing 1 nM DHT with or without test compounds. For all experiments, fresh medium containing the test compounds was changed daily and cells were harvested for total RNA isolation using the Trizol method (Invitrogen/Life Technologies) after 48 h. Taqman real-time PCR was used to quantify expression of the mRNA.

Small interfering RNA (siRNA) oligonucleotides studies. Small interfering RNA specifically block expression of AR or TWIST1 mRNA was used to confirm the role of androgen regulation of TWIST1, CCL2 and TNF-α mRNA expression. LNCaP cells were seeded in 6-well plates as described above. Twenty-four hrs after androgen deprivation, LNCaP cells were treated with or without 1 nM dihydrotestosterone (DHT). For siRNA experiments, cells were transfected with 5 nM of small interfering RNA oligonucleotides targeting AR or TWIST1 using HiPerFect transfection reagent according to the manufacturer's protocol. Forty-eight hours after transfection, cell culture media were replaced with fresh media in the presence or absence of 1 nM DHT for an additional 48 h. After treatment, cells were harvested for total RNA isolation as described above.

Trans-well cell migration assay. For trans-well migration assays, 2 × 10^5 THP-1 cells in 0.2 ml phenol-red free RPMI1640 (Invitrogen/Life Technologies) were placed into the non-coated membrane top
chamber (24-well insert; pore size, 8 μm; Corning Costar) and allowed to migrate toward LNCaP cells-
conditioned media (1.2 ml) in the lower chamber. After 10 hours of incubation with the conditioned media,
cells migrated to the lower chamber were collected by centrifugation (1000 g x 10 min), re-suspend in 0.1
ml PBS and the number of cells counted under a light microscope (Olympus CK40) using a
hemocytometer. To confirmed that CCL2 in conditioned media contributed toward migration of THP-1
cells, condition media were first treated with 0.5 μg/ml anti-human neutralizing CCL2 antibody (R&D
Systems) or IgG isotype control (BD Pharmingen) for 1hr , then migration assays were conducted as
above.

**Enzyme-linked immunosorbent assay (ELISA) determination of CCL2 and TNF-α protein.** LNCaP
cell were treated with or without DHT (1 nM) in presence or absence of DIM (10 μM) or I3C (50 μM) for
48 h. After incubation, LNCaP cells conditioned media was removed, lyophilized and re-suspended in
RPMI-1640 phenol red free media (Invitrogen/Life Technologies) to yield 200 μg protein/μL, 50 μl was
used for detection of CCL2 or TNF-α. Protein expressions of CCL2 and TNF-α were determined by using
commercially available ELISA kits. EH2MCP1 for CCL-2, EHTNFA for TNF-α (Thermo Scientific,
Rockford, IL, USA) according to the manufacturer's instructions. The optical density (OD) was
determined at 550 nm or 450 nm using a multi-well plate reader (Spectra Max Plus, Molecular Devices,
Sunnyvale, CA, USA).

**Statistical methods.** Statistical analysis of data was carried out with the GraphPad PRISM 4 program
(GraphPad Software, Inc., La Jolla, CA). Multiple group data were analyzed using one-way or two-way
ANOVA followed by a Bonferroni post-hoc test. An unpaired Student's t test was used to compare
experiments between two groups. Gene expression results are expressed as means ± SEM relative to
vehicle control. Data are representative of three independent experiments.
**Results**

**Androgen Induces TWIST1 mRNA Expression through an AR-dependent pathway.** DHT (0-1nM) significantly induced TWIST1 in a dose and time-dependent manner (Fig. 1A and B). As shown in Fig. 1C and D, adding the AR siRNA effectively inhibited DHT induction of the well-documented androgen responsive gene PSA (Fig. 1C). Similarly, AR siRNA also inhibited DHT induction of TWIST-1 mRNA (1D).

**Androgen differentially Regulate putative TWIST-1 Down-Stream Target genes mRNA**

**Expression.** DHT- treatment of LNCaP cells lead to differential effects on TWIST-1-responsive gene mRNA expression. EMT-related, TWIST-1-responsive genes such as, CDH1, SNAIL and VIM were not affected by DHT treatment (Fig. 2A, B and C), and the expression of DES and CDH2 was below detection limits under the current experimental condition. By contrast, CCL2 and TNF, two inflammation-related putative TWIST-1-responsive genes, were induced by DHT treatment in a time- and dose-dependent manner (Fig. 2D to G).

**Androgen Regulation of CCL2 and TNF-α mRNA is through an AR but not a TWIST-1 dependent pathway.** Both androgen receptor and TWIST1 may both contribute to the up-regulation of CCL2 and TNF mRNA levels by DHT-treatment. Small interfering RNA against AR effectively inhibited DHT (1nM) induced increase in CCL2 and TNF-α mRNA levels (Fig. 3A, B). In the contrast, siRNA against TWIST1 did not affect the expressions of DHT-induction of CCL2 or TNF (Figure 3 C, D). Treatment of cells with siRNA for AR and TWIST1 lead to ~90% and ~ 80% inhibition of AR and TWIST expression, respectively (Figure 3E). TWIST1, CCL2 and TNF-α mRNA expression levels were higher in PC-3 cells than in LNCaP cells (Fig. 4A), Furthermore, treatment of PC-3 cells with DHT (1 nM) did not affect the mRNA expression levels of these genes (Fig. 4B, C). Consistent with increase in CCL2 mRNA levels, DHT also lead to an increase in CCL2 protein levels (Fig. 5A)

**Conditioned Media from DHT-treated LNCaP cell Increase THP-1 Monocyte Migration.** As shown in Fig. 5B, the number of THP-1 cells migrated toward DHT-treated LNCaP cells was significantly higher than in those provided the control media. Adding DHT (1 nM) directly into media did not lead to
increase in migration of THP-1 cells. Pre-treatment of conditioned media with a neutralizing antibody against CCL2 was found to attenuate increased migration of THP-1 cells toward DHT-treated conditioned media (Fig. 5C).

**I3C and DIM Modulate Effects of Androgen on CCL2 and Migration.** Consistent with their effect on androgen-dependent pathways, I3C and DIM exerted a concentration-dependent inhibition of both TWIST1 and CCL2 mRNA expression induced by DHT treatments. This response was similar to the effects of I3C and DIM had on androgen induction of the well-documented androgen responsive gene PSA mRNA (Fig. 6A, B and C). Furthermore, treatment of cells with I3C (50 μM) and DIM (10 μM) also inhibited the DHT-induced increase in CCL2 protein (Fig. 6D and E). Furthermore, I3C (50 μM) or DIM (10 μM) treatment also led to significantly reduction of DHT-induced migration of THP-1 cells (Fig. 6F and G).
Discussion

The molecular mechanisms underlying prostate cancer’s etiology and progression remain unclear. Although exposure to androgen is consider one of the risk factor for prostate cancer, the mechanisms also not completely delineated. In the present study we reported a novel finding that CCL2, a protein critical in attracting monocytes (37,38), is an androgen-responsive gene. We show that exposure of the androgen-responsive LNCaP cell line to DHT led to induction of CCL2 mRNA as well as protein (Figure 5A) and this effect of DHT on CCL2 mRNA was blocked by siRNA against AR (Figure 3A). This observation supports the notion that exposure to androgens can promote attraction of monocytes by prostate cancer cells. We provide in-vitro data (Figure 5C) to support this notion as we observed increased migration of THP-1 toward conditioned media derived from DHT-treated cells (Fig. 5B). The involvement of CCL2 was further strengthened, because THP-1 cell migration was inhibited when conditioned media was pre-incubated with anti-CCL-2 antibody (Fig. 5C). These results suggest that exposure of prostate cancer cells to androgens may create a pro-inflammatory environment, where the prostate cancer cell is stimulated to secrete CCL2 and attract monocytes toward tumor site. Monocytes can produce cytokines such as IL-6 and IL-1β (39). These cytokines are known to stimulate prostate cancer cell growth and angiogenesis (40). Therefore, up-regulation of CCL2 may be a mechanism whereby androgens promotes prostate cancer development. We also observed that the androgen-independent prostate cancer cell PC-3 express higher levels of CCL2 mRNA. PC-3 cell is considered to be more advance than LNCaP cell in prostate cancer tumorigenesis process. The higher expression of CCL2 in PC-3 cells as compared to LNCaP cells is consistent with PC-3’s more aggressive phenotype, and suggest that they may have a grater capacity for monocyte recruitment. This will lead to increased inflammation in the tumor site. One may thus reason that anti-inflammatory agents targeting the CCL2 pathway may delay tumor growth in both androgen-dependent and -independent prostate tumors.

One of our initial hypotheses was that DHT could modulate EMT. Epithelial-mesenchymal transition induction in cancer cells results in the acquisition of invasive and metastatic properties. Epithelial-mesenchymal transition -type cells, which share molecular characteristics with cancer stem cells (CSCs),
are believed to play critical roles in early cancer metastasis and drug resistance as demonstrated in several human malignancies including prostate cancer (41-43). We found that LNCaP cells exposed to DHT significantly up-regulated the EMT-related protein TWIST1 mRNA levels. This effect of DHT on TWIST1 is dependent on AR as siRNA against AR blocked the induction of TWIST1 mRNA by DHT (Fig. 1). TWIST1, a helix-loop-helix transcription factor, is highly expressed in many types of human cancers (44). Recently, TWIST-1 was suggested to be an oncogene (45-47) and confers prostate cancer cells with an enhanced metastatic potential through promoting EMT and a high TWIST1 expression in human prostate cancer is associated with an increased metastatic potential (48). However, we did not find DHT to effect the TWIST-1-regulated and/or EMT-related genes such as CDH1, SNAIL and vimentin expression in LNCaP cells (Fig. 2A-C). These results suggest that although androgens induced TWIST-1, they did not lead to development of an EMT phenotype in our experimental conditions. TWIST1 was also reported to be a regulator of CCL2 (49). We did found DHT treatment increased the mRNA of two purported TWIST-1 responsive genes, CCL2 and TNF-α. TNF-α is also a potent chemoattractant for several cell lines (50). However, the effect of androgen on CCL2 and TNF-α mRNA appeared to be AR-dependent pathway but not TWIST1 dependent. Androgen receptor siRNA and not TWIST1 siRNA blocked the effects of DHT on CCL2 and TNF-α mRNA. These results suggest that TWIST1 is not involved in the regulation of CCL2 or TNF-α mRNA by DHT in LNCaP cells. Additional studies are necessary to elucidate the role of DHT in EMT as well as TWIST1 in the prostate cancer/EMT relationship.

We previously reported I3C and DIM can inhibit LNCaP cell growth that correlated with inhibition of androgen-dependent pathways (33). Consistent with this observation, cells treated with I3C and DIM also significantly blocked the induction of CCL2 mRNA by DHT (Fig. 6C). In addition, I3C and DIM treatment also led to the inhibition of CCL2 protein expression induced by DHT as well as migration of THP-1 monocytes induced by DHT (Fig. 6F,G). These results indicate that exposure of cells to I3C and DIM not only modulates the growth of androgen responsive prostate cancer cells but also indirectly influences luminal cells such as the monocytes. We reason that I3C and DIM, through inhibition of
monocyte to the tumor site, could minimize the exposure of tumors to cytokines such as IL-1β and IL-6. This effect may thus attenuate the inflammatory process and ultimately lead to delay in prostate tumor growth and angiogenesis. Our data also support the possibility that diet or diet-derived compounds may act through multiple mechanisms including inflammation and angiogenesis to prevent prostate cancer. Additional in-vivo studies are warranted to validate our hypothesis. TWIST1, an androgen responsive gene, was also modulated by I3C and DIM (Figure 6B). The consequence of TWIST1 modulation by I3C and DIM remain unclear and warrant further study.

In summary, we found that the androgen DHT promotes monocyte migration through up-regulation of CCL2 mRNA and protein. The effect of DHT on CCL2 appeared to be independent of TWIST-1 and mediated through AR-dependent pathways. More importantly the diet-derived factors I3C and DIM can modulate CCL2 and monocyte migration induced by DHT. These may be important mechanisms whereby diet-derived compound delay prostate tumor growth and development. Finally, we found that DHT induced EMT-related TWIST-1 expression but had no effect on other EMT-related genes downstream of TWIST-1.
References


Figure legends

Figure 1. Effects of DHT on TWIST-1 expression in androgen-responsive LNCaP cells. LNCaP cells (0.25×10^6 cells/well) were plated in six-well plates, after androgen deprivation, the cells were daily treated with vehicle (DMSO) or DHT, and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control (*P < 0.05, **P < 0.01, ***P < 0.001). A. Concentration dependent effects of DHT on TWIST-1 mRNA levels. The cells were treated daily with or without varied concentrations (0-1.0 nM) of DHT for 48 h. B. Time course of DHT effects on TWIST1 mRNA levels. LNCaP cells were daily treated with or without 1 nM DHT for 0, 24, 48, or 72 h. C, D. Effects of AR siRNA on TWIST-1 and PSA mRNA levels. LNCaP cells were transfected with 5 nM AR siRNA or Negative control siRNA as described in the Materials and Methods. After transfection, the cells were treated with or without DHT (1nM) for 48 h as described in Materials and Methods. After treatment, total RNA was isolated and mRNA for PSA and TWIST-1 determined as described in Materials and Methods. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control (*P < 0.05, **P < 0.01, ***P < 0.001). C. Effect of AR siRNA on DHT induction of TWIST1 mRNA levels. D. Effect of AR siRNA on DHT induction of PSA mRNA levels. NC: negative control siRNA.

Figure 2. Effects of DHT on TWIST1-related genes expression in androgen-responsive LNCaP cells. LNCaP cells (0.25×10^6 cells/well) were plated in six-well plates, after androgen deprivation, the cells were daily treated with vehicle or DHT for 48 h, and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control (*P < 0.05, **P < 0.01, ***P < 0.001).
A. Concentration dependent effects of DHT on CDH1 mRNA levels. B. Concentration dependent effects of DHT on SNAIL mRNA levels. C. Concentration dependent effects of DHT on vimentin mRNA levels. D. Concentration dependent effects of DHT on CCL2 mRNA levels. E. Concentration dependent effects of DHT on TNF-α mRNA levels. F. Time course of DHT effects on CCL2 mRNA levels. G. Time course of DHT effects on TNF-α mRNA levels.

Figure 3. Effects of siRNA against AR and TWIST-1 on DHT-induction of CCL2 and TNF-α mRNA expression in LNCaP cells. LNCaP cells were transfected with 5 nM AR siRNA, TWIST-1 siRNA or Negative control siRNA as described in the Materials and Methods. After transfection, the cells were treated with or without DHT (1nM) for 48 h as described in Materials and Methods. After treatment, total RNA was isolated and mRNA for AR, TWIST-1, CCL-2 and TNF- α determined as described in Materials and Methods. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control (*P < 0.05, **P < 0.01, ***P < 0.001). A. Effects of AR siRNA on DHT-induction of CCL2 mRNA expression. B. Effects of AR siRNA on DHT-induction of TNF-α mRNA expression. C. Effects of TWIST-1 siRNA on DHT-induction of CCL2 mRNA expression. D. Effects of TWIST-1 siRNA on DHT-induction of TNF-α mRNA expression. E. Effects of AR or TWIST-1 siRNA on AR (left panel) and TWIST-1 (right Panel) mRNA expression. NC: negative control siRNA.

Figure 4. Baseline and DHT-induced mRNA levels of TWIST-1, CCL-2 and TNF-α in androgen-responsive LNCaP and non-responsive PC-3 cells. LNCaP or PC-3 cells were plated in six-well plates, after androgen deprivation, the cells were daily treated with vehicle or DHT for 48 h, and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control.
Relative baseline mRNA levels of TWIST-1, CCL-2 and TNF-α in LNCaP and PC-3 cells. B. Effect of DHT on genes expression in androgen-responsive LNCaP cells. C. Effects of DHT on genes expression in androgen-nonresponsive PC-3 cells.

Figure 5. Effect of DHT on CCL2 protein and LNCaP cell conditioned media-stimulated THP-1 monocyte migration. LNCaP cells were treated with or without 1 nM DHT for 48 h, conditioned media harvested and CCL2 protein level, THP-1 migration assay performed as described in Materials and Methods. The results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments. Error bars with an asterisk are significantly different than vehicle-treated control (**P <0.01, ***P < 0.001, as analyzed by Bonferroni post-test). A. Protein expressions of CCL2 in LNCaP cells conditioned media. Error bars with an asterisk are significantly different than vehicle-treated control (**P <0.01, ***P < 0.001). B. Effect of conditioned media on THP-1 cells migration. Error bars with an asterisk are significantly different than vehicle-treated control (**P <0.01, ***P < 0.001). C. Effect of CCL2 Blocking antibody on conditioned media-induced THP-1 migration. LNCaP cells conditioned media were treated with 0.5 μg/ml isotype control or CCL2 blocking antibody for 1 h then the THP-1 migration assay was conducted as described in Materials and Methods. Values with a different superscript are significantly different at p<0.05.

Figure 6. Effect of I3C and DIM on DHT-induced gene expression in LNCaP cells and conditioned media stimulated THP-1 cell migration. LNCaP cells were treated with or without 1 nM DHT for 48 h, cell harvested for total RNA isolation and gene expression analysis as described in Materials and Methods. Conditioned media were harvested for CCL2 protein ELISA and THP-1 migration assay as described in Materials and Methods. The results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments. A. I3C and DIM inhibition of DHT-induced PSA expression. B. I3C and DIM inhibition of DHT-induced TWIST-1 mRNA expression. C. I3C and DIM inhibition of DHT-induced CCL2 mRNA expression. D. I3C repressed the protein expression of CCL2 in DHT-treated
LNCaP media. E. DIM repressed the protein expression of CCL2 in DHT-treated LNCaP media. F. I3C suppressed THP-1 migration in DHT-treated LNCaP conditioned media. G. DIM suppressed THP-1 migration in DHT-treated LNCaP conditioned media. Error bars with an asterisk are significantly different than vehicle-treated control (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 5A

CCL2 (pg/mL)

Con  | DHT
---   | ---
0     | 60

Figure 5B

Cell # \(1 \times 10^6\)

Con  | DHT
---   | ---
0     | 12.5

Figure 5C

Cell migrated \(\times 10^4\)/ml

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