Research Article

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 was found to induce a time-dependent (0–72 hours) and concentration-dependent (0–1 nmol/L) 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 dihydrotestosterone was mediated through an androgen receptor (AR)-dependent pathway as small inhibitor RNA against AR negated the induction of CCL2. Although dihydrotestosterone 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 dihydrotestosterone 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 dihydrotestosterone on CCL2 and migration. These results show that androgen may regulate CCL2 and contribute to the inflammatory microenvironment in prostate tumors and that this process can be blocked by broccoli-derived compounds.

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Introduction

Androgens are functionally required for the normal growth and development of the prostate gland. In adult males, androgens promote secretory 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 that many of the biologic effects of androgen are likely through the regulation of androgen-responsive genes (ARG) via an androgen receptor (AR)–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 that 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 biologic pathways associated with EMT. Androgens were reported to induce changes that are characteristic of EMT such as expression of TWIST1, CDH1, and SNAI1; cytoskeleton reorganization; and promotion of metastatic behavior of castration-resistant prostate cancer cells (20). TWIST1, a helix-loop-helix transcription factor thought to be an important mediator of EMT, is upregulated in several types of epithelial cancers including prostate, breast, and gastric carcinomas (21–24). Several EMT-related genes, such as CDH1, CDH2, SNAI1, desmin, and vimentin, are putative downstream targets regulated by TWIST1. TWIST1 also appears to be involved in inflammatory pathways (25). TWIST1 expression in T-helper 1 (Th1) lymphocytes and bone marrow–derived macrophages attenuates the expression of IFN-γ, interleukin (IL)-2, and TNF-α, further implicating TWIST1 in the regulation of cytokine expression (25–27). It was also reported that CCL2 expression may be subject to regulation by TWIST1 (28). The relationship between androgens and these EMT-related proteins as well as the relation between TWIST1 and CCL2 in prostate cells remains 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 prevent or delay the progression of cancer, through their ability to attack cancer stem cells or EMT-type cells and promote metastatic behavior of castration-resistant cancer and EMT and existent information on TWIST1 remain unknown.

Given the potential roles of androgen in regulating prostate cancer and EMT and existent information on TWIST1 remain unknown.

Materials and Methods

Chemicals and reagents

I3C, DIM, dihydrotestosterone (DHT), and dimethyl sulfoxide (DMSO) were from Sigma Chemical. TRIzol, AffinityScript Multiple Temperature cDNA Synthesis kit, and TaqMan real-time PCR primers and probes were obtained from Life Technology. On-Target plus SMART pool siRNA targeting AR and TWIST1 were purchased from Thermo Fisher Scientific. HiPerFect Transfection Reagent was purchased from Qiagen.

Cell culture

LNCaP and PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (ATCC) and maintained in medium A [RPMI-1640 medium with phenol red and 2 mmol/L L-glutamine (Invitrogen/Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin (BioSource International) with 10% FBS (Invitrogen/Life Technologies)]. For androgen-related experiments, cells were seeded as follows: 6-well plates at 250,000 cells/well. After 24 hours in medium A, cells were switched to medium B, which had the same composition as medium A except that FBS was replaced with 10% charcoal dextran-treated FBS (GIBCO/Life Technologies) for an additional 24 hours 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 ATCC and maintained in RPMI-1640 medium with phenol red and 2 mmol/L L-glutamine (Invitrogen/Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin (BioSource International) with 10% FBS (Invitrogen/Life Technologies).

RNA isolation and real-time reverse transcription PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen/Life Technologies) 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 seconds, followed by 46 cycles of amplification at 95°C for 1 second and 60°C for 30 seconds. Relative mRNA fold changes to control were calculated using the comparative Ct 2−ΔΔCt 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 (SNAI1), DES (desmin), VIM (vimentin), KLK3 (PSA), CCL2, or TNF (TNF-α) mRNA levels, LNCaP cells were plated in 6-well plates (0.25 × 10^6 cells/well) in medium A, and after 24 hours, the media was removed and replaced with fresh media containing vehicle or varied concentrations of test compounds I3C at 5, 25, 50 μmol/L and DIM at 1, 5, 10 μmol/L. LNCaP cells were plated in 6-well plates (0.25 × 10^6 cells/well) in medium A and switched to medium B 24 hours after plating to minimize the effect of serum hormones. The cells were then incubated in medium B for an additional 24 hours before the treatments began. Twenty-four hours later, the medium was replaced with fresh medium containing 1 nmol/L dihydrotestosterone 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 hours. TaqMan real-time PCR was used to quantify expression of the mRNA.

siRNA oligonucleotide studies
siRNAs 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 hours after androgen deprivation, LNCaP cells were treated with or without 1 nmol/L dihydrotestosterone. For siRNA experiments, cells were transfected with 5 nmol/L of siRNA 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 nmol/L dihydrotestosterone for an additional ours. After treatment, cells were harvested for total RNA isolation as described above.

Transwell cell migration assay
For transwell migration assays, 2 × 10⁵ THP-1 cells in 0.2 mL phenol-red free RPMI-1640 (Invitrogen/Life Technologies) were placed into the noncoated membrane top chamber (24-well insert; pore size, 8 μm; Corning Costar) and allowed to migrate toward LNCaP cell-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 (1,000 rpm for 10 minutes), resuspended 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 medium 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 1 hour and then migration assays were conducted as above.

ELISA determination of CCL2 and TNF-α protein
LNCaP cell were treated with or without dihydrotestosterone (1 nmol/L) in presence or absence of DIM (10 μmol/L) or I3C (50 μmol/L) for 48 hours. After incubation, LNCaP cells conditioned media were removed, lyophilized, and resuspended 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) were used according to the manufacturer’s instructions. The optical density (OD) was determined at 550 or 450 nm using a multiwell plate reader (Spectra Max Plus, Molecular Devices).

Statistical methods
Statistical analysis of data was conducted with the GraphPad Prism 4 program (GraphPad Software, Inc.). Multiple group data were analyzed using 1- or 2-way ANOVA followed by a Bonferroni post hoc test. An unpaired Student t test was used to compare experiments between 2 groups. Gene expression results are expressed as means ± SEM relative to vehicle control. Data are representative of 3 independent experiments.

Results
Androgen induces TWIST1 mRNA expression through an AR-dependent pathway
Dihydrotestosterone (0–1 nmol/L) 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 dihydrotestosterone induction of the well-documented androgen responsive gene PSA (Fig. 1C). Similarly, AR siRNA also inhibited dihydrotestosterone induction of TWIST1 mRNA (Fig. 1D).

Androgen differentially regulate putative TWIST1 downstream target genes mRNA expression
Dihydrotestosterone treatment of LNCaP cells led to differential effects on TWIST1-responsive gene mRNA expression. EMT-related, TWIST1-responsive genes such as, CDH1, SNAIL, and VIM were not affected by dihydrotestosterone treatment (Fig. 2A–C), and the expression of DES and CDH2 was below detection limits under the current experimental condition. In contrast, CCL2 and TNF, 2 inflammation-related putative TWIST1-responsive genes, were induced by dihydrotestosterone treatment in a time- and dose-dependent manner (Fig. 2D–G).

Androgen regulation of CCL2 and TNF-α mRNA is through an AR but not a TWIST1-dependent pathway
Both AR and TWIST1 may both contribute to the upregulation of CCL2 and TNF mRNA levels by dihydrotestosterone treatment. siRNA against AR effectively inhibited dihydrotestosterone (1 nmol/L)-induced increase in CCL2 and TNF-α mRNA levels (Fig. 3A and B). In contrast, siRNA against TWIST1 did not affect the expressions of dihydrotestosterone induction of CCL2 or TNF (Fig. 3C and D). Treatment of cells with siRNA for AR and TWIST1 lead to about 90% and 80% inhibition of AR and TWIST expression, respectively (Fig. 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 dihydrotestosterone (1 nmol/L) did not affect the mRNA expression levels of these genes (Fig. B and C). Consistent with increase in CCL2 mRNA levels, dihydrotestosterone also lead to an increase in CCL2 protein levels (Fig. 5A)

Conditioned media from dihydrotestosterone-treated LNCaP cell increase THP-1 monocyte migration
As shown in Fig. 5B, the number of THP-1 cells migrated toward dihydrotestosterone-treated LNCaP cells was significantly higher than in those provided the control media. Adding dihydrotestosterone (1 nmol/L) directly into media did not lead to increase in migration of THP-1 cells.
Pretreatment of conditioned media with a neutralizing antibody against CCL2 was found to attenuate increased migration of THP-1 cells toward dihydrotestosterone-treated conditioned media (Fig. 5C).

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 dihydrotestosterone treatments. This response was similar to the effects that I3C and DIM had on androgen induction of the well-documented androgen-responsive gene PSA mRNA (Fig. 6A–C). Furthermore, treatment of cells with I3C (50 μmol/L) and DIM (10 μmol/L) also inhibited the dihydrotestosterone-induced increase in CCL2 protein (Fig. 6D and E). Furthermore, I3C (50 μmol/L) or DIM (10 μmol/L) treatment also led to significantly reduction of dihydrotestosterone-induced migration of THP-1 cells (Fig. 6F and G).

**Discussion**

The molecular mechanisms underlying the etiology and progression of prostate cancer remain unclear. Although exposure to androgen is considered as one of the risk factor for prostate cancer, the mechanisms are also not completely delineated. In the present study, we reported a novel finding that CCL2, a protein critical in attracting
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 6-well plates, and after androgen deprivation, the cells were daily treated with vehicle or DHT for 48 hours 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 3 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.
monocytes (37, 38), is an androgen-responsive gene. We show that exposure of the androgen-responsive LNCaP cell line to dihydrotestosterone led to induction of CCL2 mRNA as well as protein (Fig. 5A), and this effect of dihydrotestosterone on CCL2 mRNA was blocked by siRNA against AR (Fig. 3A). This observation supports the notion that exposure to androgens can promote attraction of monocytes by prostate cancer cells. We provide in vitro data (Fig. 5C) to support this notion as we observed increased migration of THP-1 toward conditioned media derived from dihydrotestosterone-treated cells (Fig. 5B). The involvement of CCL2 was further strengthened because THP-1 cell migration was inhibited when conditioned media were preincubated 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

Figure 3. Effects of siRNA against AR and TWIST1 on DHT induction of CCL2 and TNF-α mRNA expression in LNCaP cells. LNCaP cells were transfected with 5 nmol/L AR siRNA, TWIST1 siRNA, or negative control siRNA as described in the Materials and Methods. After transfection, the cells were treated with or without DHT (1 nmol/L) for 48 hours as described in Materials and Methods. After treatment, total RNA was isolated and mRNA for AR, TWIST1, 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 3 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 TWIST1 siRNA on DHT induction of CCL2 mRNA expression. D, effects of TWIST1 siRNA on DHT induction of TNF-α mRNA expression. E, effects of AR or TWIST1 siRNA on AR (left) and TWIST1 (right) mRNA expression. NC, negative control siRNA.
Therefore, upregulation of CCL2 may be a mechanism whereby androgens promote prostate cancer development. We also observed that the androgen-independent prostate cancer cell PC-3 expresses higher levels of CCL2 mRNA. PC-3 cell is considered to be more advanced than LNCaP cell in prostate cancer tumorigenesis process. The higher expression of CCL2 in PC-3 cells than in LNCaP cells is consistent with the more aggressive phenotype of PC-3 and suggests that they may have a greater 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 dihydrotestosterone could modulate EMT. EMT induction in cancer cells results in the acquisition of invasive and metastatic properties. EMT-type cells, which share molecular characteristics with cancer stem cells (CSC), are believed to play critical roles in early cancer metastasis and drug resistance as shown in several human malignancies including prostate cancer (41–43). We found that LNCaP cells exposed to dihydrotestosterone significantly upregulated the EMT-related protein TWIST1 mRNA levels. This effect of dihydrotestosterone on TWIST1 is dependent on AR as siRNA against AR blocked the induction of TWIST1 mRNA by dihydrotestosterone (Fig. 1). TWIST1, a helix-loop-helix transcription factor, is highly expressed in many types of human cancers (44). Recently, TWIST1 was suggested to be an oncogene (23, 45, 46) 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 (47). However, we did not find dihydrotestosterone to effect the TWIST1-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 TWIST1, they did not lead to development of an EMT phenotype in our experimental conditions. TWIST1 was also reported to be a regulator of CCL2 (48). We did found that dihydrotestosterone increased the mRNA of 2 purported TWIST1-responsive genes, CCL2 and TNF-α. TNF-α is also a potent chemoattractant for several cell lines (49). However, the effect of androgen on CCL2 and TNF-α mRNA appeared to be AR-dependent pathway but not TWIST1-dependent. AR siRNA and not TWIST1 siRNA
blocked the effects of dihydrotestosterone on CCL2 and TNF-α mRNA. These results suggest that TWIST1 is not involved in the regulation of CCL2 or TNF-α mRNA by dihydrotestosterone in LNCaP cells. Additional studies are necessary to elucidate the role of dihydrotestosterone in EMT as well as TWIST1 in the prostate cancer–EMT relationship.

We previously reported that 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 dihydrotestosterone in LNCaP cells. Additional studies are necessary to elucidate the role of dihydrotestosterone in EMT as well as TWIST1 in the prostate cancer–EMT relationship.

In summary, we found that the androgen dihydrotestosterone promotes monocyte migration through upregulation of CCL2 mRNA and protein. The effect of dihydrotestosterone on CCL2 appeared to be independent of TWIST1.
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 nmol/L DHT for 48 hours, 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 3 separate experiments. A, I3C and DIM inhibition of DHT-induced PSA expression. B, I3C and DIM inhibition of DHT-induced TWIST1 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).
and mediated through AR-dependent pathways. More importantly, the diet-derived factors I3C and DIM can modulate CCL2 and monocyte migration induced by dihydrotestosterone. These may be important mechanisms whereby diet-derived compound delay prostate tumor growth and development. Finally, we found that dihydrotestosterone induced EMT-related TWIST1 expression but had no effect on other EMT-related genes downstream of TWIST1.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: E.-K. Kim, Y.S. Kim, J.A. Milner, T.Y.T. Wang
Development of methodology: E.-K. Kim, T.Y.T. Wang

References
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