Androgen-Induced Wnt Signaling in Preosteoblasts Promotes the Growth of MDA-PCa-2b Human Prostate Cancer Cells

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Abstract

The high morbidity and mortality associated with prostate cancer (PCa) result from its tendency to metastasize to bone where it produces predominantly osteoblastic lesions. The Wnt signaling pathway plays an important role in embryogenesis, tumorigenesis, osteoblast development, and bone formation. Androgen signaling via the androgen receptor (AR) is critical in both PCa and bone cell growth. We examined the effects of androgens on cell growth and Wnt signaling in the AR-positive MDA-PCa-2b cell line and MC3T3 preosteoblasts, grown alone and in coculture. We show that the potent androgen dihydrotestosterone increases AR expression and transcriptional activity only in the preosteoblasts. Although dihydrotestosterone induced an 80% increase in PCa cell growth when the cells were grown alone, dihydrotestosterone had a more significant effect on MDA-PCa-2b cell proliferation (3.2-fold increase) when the PCa cells were cocultured with preosteoblasts. Dihydrotestosterone addition to preosteoblasts promoted Wnt-dependent transcriptional reporter activity associated with GSK3β-9 phosphorylation and accumulation of nuclear β-catenin as well as elevated Runx2 expression. In addition, the increased proliferation of PCa cells in coculture with MC3T3 cells in response to dihydrotestosterone was abrogated by the addition of either exogenous DKK-1 or sFRP-1 protein, two naturally occurring Wnt antagonists. Finally, we show that the paracrine growth-promoting effect of androgens is limited to MDA-PCa-2b cells. These data imply that Wnt signaling is involved in the androgen-regulated crosstalk between preosteoblasts and PCa cells and suggest that androgens may stimulate growth of some prostate tumor cells indirectly, via up-regulation of Wnt signaling in bone cells. [Cancer Res 2007;67(12):5747–53]

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer deaths in U.S. males. The high morbidity and mortality associated with PCa derive from its tendency to metastasize to bone (1). Although there is evidence that PCa initially induces osteolytic lesions (2), PCa-induced bone metastases are unique in that they typically produce an osteoblastic reaction in bone (3). The precise mechanisms underlying this tendency for PCa cells to target bone and induce an osteoblastic response in the bone microenvironment have not been well delineated. These bony metastases grow more rapidly than primary or other metastatic lesions (3, 4), suggesting that interactions between PCa cells and the bone microenvironment may promote PCa growth and progression.

PCa cell growth in both the primary site and the bone microenvironment is under both autocrine regulation involving androgen-mediated up-regulation of several growth factors (5, 6) and paracrine influences involving the stromal components (4, 7). Clinically, a bidirectional interaction between PCa cells and osteoblasts has been shown to stimulate both bone targeting and bone reaction (4). Several factors, such as basic fibroblast growth factor (4), osteocalcin, bone sialoprotein (8), and interleukin-6 (9), have been shown to be responsible for this pattern of PCa progression. However, the precise events and underlying mechanisms have not been fully elucidated.

The Wnt signaling family includes 19 secreted glycoproteins that have functions related to embryogenesis, cell specification, formation of the body plan, cell growth, differentiation, and apoptosis (10). Wnt signaling plays a central role in osteoblast development and bone formation (11). Wnts promote the lineage commitment of mesenchymal precursor cells and the differentiation of progenitor cell lines into osteoblasts (11, 12). In addition, they stimulate osteoblast maturation and exert direct effects on the formation and turnover of the mature skeleton. Wnts directly stimulate tumor cell growth and survival via autocrine regulation in several types of human cancer, including PCa (13).

Androgen signaling via the androgen receptor (AR) is a key pathway that contributes to PCa progression (14, 15). Androgens also exert direct anabolic effects on normal bone (16, 17). Recent studies show that there is significant crosstalk between the Wnt/β-catenin and AR signaling pathways. Functional colocalization of AR, β-catenin, and Tcf in the nucleus has been reported (18, 19). It has also been shown that β-catenin preferentially binds AR over several other steroid hormone receptors, including the estrogen receptor, progesterone receptor, and glucocorticoid receptor (18). Moreover, forced overexpression of β-catenin augments AR-mediated transcription of several AR-regulated promoters in both prostate and nonprostate cells (19, 20), indicating that β-catenin may act as a coactivator of AR and that AR may require β-catenin to regulate gene expression. Although increasing evidence has shown an interaction between AR and Wnt signaling in PCa cells, the possible crosstalk between the two pathways in the bone microenvironment in the setting of PCa bone metastases has not previously been well delineated.

In the present study, we examined the effects of androgens on cell growth, Wnt signaling activity, and the expression of various components of the Wnt signaling pathway in MDA-PCa-2b, an AR-positive PCa cell line, versus MC3T3, an osteoblastic precursor cell line. Our results reveal an involvement of Wnt signaling in androgen-regulated crosstalk between preosteoblasts and PCa cells.
and indicate that androgens may stimulate MDA-PCa-2b cell proliferation in the bone microenvironment indirectly via modulation of Wnt signaling in the bone cells.  

Materials and Methods

**Cell culture and reagents.** The AR-positive PCa cell line, MDA-PCa-2b, the AR-negative PC-3 cell line, and the MC3T3-E1 osteoblast precursor cell line were obtained from the American Type Tissue Collection. The PC-3M1L human PCa cell line, a subline of the PC-3 cell line (21), was a generous gift from Dr. M. Stearns (Department of Pathology, MCP-Hahnemann University, Philadelphia, PA). Cells were cultured in DMEM containing 10% fetal bovine serum. For coculture experiments, MDA-PCa-2b cells were seeded in 12-well cluster culture plates, and MC3T3 cells were seeded in cell-culture inserts with 0.4-μm pores (BD Inc.) as described previously (22). All experiments were done in triplicate and repeated three separate times. Dihydrotestosterone was purchased from Sigma. Recombinant DKK-1 and sFRP-1 proteins were purchased from R&D systems. Enhanced Luciferase Assay kit and β-catenin antibodies were obtained from BD PharMingen. Antibodies against AR, DKK-1, sFRP-1, and β-actin were purchased from Santa Cruz Inc.. Antibodies against GSK3β and phosphor-GSK3β were obtained from Cell Signaling Technology. Rux2 antibodies were purchased from MBL Co.

**Protein isolation and immunoblotting.** Cells cultured under the indicated conditions were lysed, and total protein was isolated as described previously (23). Proteins from the cytosolic and nuclear fractions were isolated using a commercial kit purchased from Pierce, according to the manufacturer’s instructions. Protein content was assayed using a kit from Bio-Rad. Western blotting was done as previously described (23). β-actin and histone H1 (for nuclear protein) were used as the internal control in Western blot analyses.

**Transient transfection and lucinereporter assay.** Transient transfection was done using LipofectAMINE 2000 reagent according to the manufacturer’s instruction (Invitrogen). The Tcf luciferase reporter construct pGL3-OT and control vector were generously provided by Dr. B. Vogelstein (John Hopkins Oncology Center, Baltimore, MD). Trans-Lucent AR Reporter Vector and control plasmid were purchased from Panomics Inc. Wnt5a expression vector was constructed as described previously (24). Cells were cultured in 12-well cluster plates and transfected with either 1 μg of the reporter plasmid or empty vector as a mock control. Internal normalization was done by cotransfection of the β-galactosidase expression vector (BD ClonTech). After 40 h, the transfected cells were lysed by scraping into reporter buffer (BD ClonTech), total protein concentration was determined, and luciferase and β-galactosidase activities were assayed and quantitated using a TD-20e Luminometer. The resulting activities were normalized to protein concentrations and β-galactosidase activity.

**Immunofluorescence assay.** Cells were incubated on glass coverslips and treated with either vehicle or dihydrotestosterone (10−8 mol/L) for 24 h. Immunofluorescence assay was done as previously reported (23). Secondary antibodies conjugated to fluorophores were used at a 1:100 dilution and were incubated for 1 h at 37°C followed by 3× 10-min washes. Positively stained cells were evaluated with a fluorescent microscope.

**Alkaline phosphatase assay.** Cultured cells were washed with PBS and sonicated in 10 mmol/L of Tris-HCl buffer (pH, 7.5) containing 0.1% Triton X-100. ALP activity in the lysate was assayed by the hydrolysis of p-nitrophenyl phosphate (Sigma) to p-nitropheno. Absorbance was determined at 405 nm and compared with a p-nitropheno (Sigma) standard titration curve. ALP activity was normalized to total protein content.

**Statistical analysis.** All results are given as mean ± SE. The effects induced by the various treatments were compared with untreated control cells using paired Student’s t test with the Bonferroni adjustment for the comparison of multiple groups. A P value of <0.05 was considered significant.

**Results**

**Dihydrotestosterone increases MDA-PCa-2b cell proliferation when cocultured with preosteoblasts.** We initially examined the effects of dihydrotestosterone on MDA-PCa-2b cells grown either alone or in coculture with MC3T3 preosteoblasts. As shown in Fig. 1, dihydrotestosterone had a modest, stimulatory effect on MDA-PCa-2b cell growth when cultured alone (80% increase, P < 0.05). In contrast, when the PCa cells were cocultured with MC3T3 cells, dihydrotestosterone strikingly increased PCa cell numbers (3.2-fold). Of note, MDA-PCa-2b cell proliferation also increased, even in the absence of dihydrotestosterone, when the PCa cells were cocultured either with MC3T3, a bone-derived fibroblast cell line, or NIH3T3, an embryonic fibroblast cell line. This androgen-independent effect of coculture with stromal cells is likely from the result of secretion of stromal-derived growth factors, as previously reported (4). Because NIH 3T3 cells do not express measurable amounts of androgen receptor, we used these cells in coculture as a negative control for androgenic effects. Our data show that dihydrotestosterone failed to induce a further increase in cell growth when the PCa cells were cocultured with NIH3T3 cells. Dihydrotestosterone had no significant direct or indirect effects on the growth of either MC3T3 or NIH3T3 cells (data not shown).

**Dihydrotestosterone induces AR expression and transcriptional activity in MC3T3 cells.** We next examined AR protein expression in MDA-PCa-2b, MC3T3, and NIH3T3 cells. As shown in Fig. 24, although MC3T3 preosteoblast cells expressed relatively low basal levels of AR, dihydrotestosterone induced a time-dependent increase in AR expression after 24 h. This AR protein induction was significant (P < 0.01), with a mean value of 3.2-fold derived from three assays. In contrast, MDA-PCa-2b cells expressed higher basal levels of AR protein, but the expression levels were not further increased by dihydrotestosterone treatment. NIH3T3 cells expressed neither basal nor inducible AR protein. AR

![Figure 1. Dihydrotestosterone induces the growth of MDA-PCa-2b cells cocultured with MC3T3 osteoblasts. Cells were cultured in serum-free medium and treated with either vehicle or 10−8 mol/L dihydrotestosterone for 7 d. The number of living cells was counted using a hemacytometer. Data are expressed as means ± SE of three wells from three separate experiments. *P < 0.01 versus the cells cultured alone without dihydrotestosterone treatment. The statistical comparisons among the other groups are shown by P value.](cancerres.aacrjournals.org)
transcriptional activity was assessed using an AR luciferase reporter assay. As shown in Fig. 2B, treatment of MC3T3 cells with dihydrotestosterone enhanced AR luciferase activity, whereas this activity was not influenced by dihydrotestosterone addition in the MDA-PCa-2b cells (Fig. 2B). These observations are in agreement with a previous study indicating that the family of MDA-PCa cell lines, including MDA-PCa-2a and -2b, exhibit very low binding affinities for dihydrotestosterone due to mutations in the ligand binding domain of the AR (25).

**Dihydrotestosterone increases Wnt signaling activity in preosteoblasts.** Functional interactions between Wnt and AR signaling in AR-positive cell lines have been shown (18–20). Thus, we tested the effects of dihydrotestosterone on the activation of the Tcf transcription factor using a Tcf-dependent luciferase transcriptional reporter. Wnt3a-transfected cells were used as a positive control for Tcf activation in both cell lines. As shown in Fig. 3, dihydrotestosterone addition resulted in a significant enhancement of Tcf-luciferase activity in MC3T3 osteoblast cells, but not in MDA-PCa-2b cells. These data show that androgens induce Wnt activity in MC3T3 preosteoblast cells but not in MDA-PCa-2b cells.

We next examined the effects of dihydrotestosterone on GSK3β protein phosphorylation using Western blot analysis with antibodies specifically against phospho-GSK3β. MC3T3 cells expressed low basal levels of phosphorylated GSK3β protein, and dihydrotestosterone significantly enhanced GSK3β phosphorylation, particularly at Ser9, in MC3T3 preosteoblast cells (Fig. 4A). This induction was statistically significant ($P < 0.01$), with a mean value of 5.0-fold derived from three assays. There was no effect of dihydrotestosterone on endogenous GSK3β protein levels in these cells. We observed no increased phosphorylation of GSK3β protein in MDA-PCa-2b cells under the same conditions (data not shown).

Activation of the Wnt signaling pathway results in the accumulation of β-catenin in the nucleus associated with heterodimer formation with Tcf and activation of Tcf-dependent transcription. Thus, we examined the effects of dihydrotestosterone on subcellular localization of β-catenin. We also examined the effect

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**Figure 2.** Dihydrotestosterone induces AR expression and transcriptional activity in MC3T3 cells. **A,** dihydrotestosterone induces AR protein expression. Cells were treated with either vehicle or dihydrotestosterone (10^{-8} mol/L) for the times indicated. Total protein was extracted and subjected to Western blotting. The same blot was stripped and reprobed with β-actin antibody. Normalized quantification (relative ratio of AR to β-actin) of the immunoblots was done by densitometry and is shown at the bottom. Data shown are representative of three separate experiments and expressed as fold induction compared with control. **B,** dihydrotestosterone induces AR transcriptional activity. Cells were cotransfected with the TransLucent AR reporter plasmid (or empty vector as mock control) and the β-galactosidase expression vector and treated with either vehicle or dihydrotestosterone (10^{-8} mol/L) for the times indicated. The resulting luciferase activity was normalized to protein concentrations and β-galactosidase activity. Data are expressed as fold induction compared with mock control (100%) and are the mean ± SE from three determinations. **,** $P < 0.01$ versus vehicle control.

**Figure 3.** Dihydrotestosterone increases Tcf-luciferase activity in osteoblasts. Cells were cotransfected with the Tcf reporter plasmid (or empty vector as mock control) and the β-galactosidase expression vector and treated with either vehicle or dihydrotestosterone (10^{-8} mol/L) for 24 h. Wnt3a-transfected cells were used as a positive control. The resulting Tcf-luciferase activities were normalized to protein concentrations and β-galactosidase activity. Data are expressed as fold induction compared with the vehicle control (100%) and represent the means ± SE from three separate determinations. **,** $P < 0.01$; **,** $P < 0.05$.
of dihydrotestosterone on nuclear Runx2 levels in MC3T3 cells because this transcription factor is a direct target gene of the Wnt signaling pathway in bone-related cell lines (26). As shown in Fig. 4B, dihydrotestosterone significantly increased both β-catenin and Runx2 nuclear levels in MC3T3 preosteoblasts. The mean values of induction derived from three assays were 3.1- and 3.0-fold (P < 0.01), respectively. The dihydrotestosterone effect on β-catenin nuclear localization was confirmed in MC3T3 cells by immunofluorescence assay (Fig. 4C). In contrast, dihydrotestosterone had no effect on the subcellular localization of β-catenin in MDA-PCa-2b cells (Fig. 4D).

Inhibition of Wnt signaling suppresses proliferative effects of dihydrotestosterone on PCA cells in coculture with preosteoblasts. In an effort to establish that dihydrotestosterone enhancement of PCA cell growth in the bone microenvironment was mediated by Wnt signaling pathway activation in preosteoblasts, we determined the effects of DKK-1 and sFRP-1, two natural inhibitors of the Wnt signaling pathway, on PCA growth in this coculture system. As shown in Fig. 5A, there was minimal effect of the inhibitors in the presence or absence of dihydrotestosterone on the growth of PCA cells when the cells were grown alone. In coculture with MC3T3, the growth stimulation observed for PCA in the absence of dihydrotestosterone was also largely unaffected by the inhibitors, implying that these effects were Wnt independent. However, both Wnt antagonists inhibited the striking increase in dihydrotestosterone-induced PCA cell growth in coculture (Fig. 5A). These findings, together with the results above, indicate that dihydrotestosterone-induced stimulation of MDA-PCa-2B cells in coculture with preosteoblasts is mediated by androgenic effects on Wnt signaling in the bone cells.

We also investigated the effects of dihydrotestosterone specifically on Tcf-Luciferase activity, cell proliferation and differentiation in MC3T3 cells in the presence or absence of Wnt inhibitors. As shown in Fig. 5B, androgen addition to single cultures of preosteoblasts increased Tcf-Luciferase activity, which was specifically inhibited by DKK-1 or sFRP-1, establishing that Wnt activation by dihydrotestosterone was responsible. Although we observed little, if any, effects of dihydrotestosterone on proliferation, alkaline phosphatase (ALP) was increased by the addition of the hormone. The inhibitors reduced both basal and dihydrotestosterone-induced
ALP levels. The above results indicated that Wnt antagonists disrupted the effects of dihydrotestosterone-stimulated Wnt activity directly in bone cells and indirectly in MDA-PCa-2b prostate cancer cells.

Both the PC-3 human prostate cancer cell line and its subline, PC-3 ML, were originally derived from human PCa bone metastases and are known to secrete high levels of DKK-1, which might be expected to abrogate the observed growth-promoting effects of coculture with preosteoblasts in the presence of dihydrotestosterone. In fact, we confirmed that androgen addition to cocultures of MC3T3 with either PC-3 or PC-3ML cells did not enhance cancer cell proliferation (Fig. 6A). To explore the mechanism underlying the differential effect of dihydrotestosterone in those PCa cell lines, we further examined the expression of Wnt inhibitors in three bone-derived PCa cell lines as well as MC3T3 cells. As shown in Fig. 6B, the expression of Wnt inhibitors was barely detectable in MC3T3 bone preosteoblasts and relatively low in MDA-PCa-2b cells. In contrast, PC-3 and PC-3ML cells expressed significantly higher levels of both DKK-1 and sFRP-1. Thus, high basal expression levels of these potent Wnt inhibitors in PC-3 and PC-3ML cells may explain the relative lack of effect of coculture with preosteoblasts in the presence or absence of dihydrotestosterone in these particular PCa cell lines.

**Discussion**

Prostate cancer (PCa) is unique in that it retains some degree of androgen sensitivity, even in its late stages. Another distinguishing feature of PCa is its tendency to metastasize to bone (1, 3) and to induce an osteoblastic reaction in the bone microenvironment (2, 3). The high morbidity and mortality associated with this disease are directly related to these bone metastases (3). Bone lesions induced by PCa cells grow at a more rapid rate than primary PCa or metastatic PCa in other sites (3, 4), suggesting that bone stromal cells provide a particularly favorable soil for PCa cell growth and progression (27) by secreting soluble growth factors (4, 28, 29). Androgens have been shown to influence normal and abnormal prostate epithelial cell growth both directly (5, 6) and,
bone matrix, it enabled us to focus specifically on androgenic microenvironment that contains endothelial cells, osteoclasts, and in vitro in the cocultures, in contrast, expresses functional AR protein.

We used an in vitro coculture system to investigate the indirect effects of androgens on PCa cell growth via interactions with preosteoblasts. We selected the MDA-PCa-2b cell line for these studies because (a) they are derived from a human PCa bone metastasis, (b) they express AR protein but have an impaired response to androgens (31), and (c) they produce an osteoblastic bone reaction in vivo (32). The MC3T3 preosteoblast cell line used in the cocultures, in contrast, expresses functional AR protein. Although this in vitro system does not mimic the full in vivo bone microenvironment that contains endothelial cells, osteoclasts, and bone matrix, it enabled us to focus specifically on androgenic modulation of preosteoblast-derived growth factors that stimulate PCa growth.

Androgens play a critical role in both PCa development and progression (14, 15). It has been shown that androgens activate growth-promoting, growth-inhibitory, and/or cell differentiation pathways directly in AR-expressing PCa cells, and the balance of these activities may depend on the stage of differentiation of the cancer cells (33). Androgens also target bone osteoblasts and osteoblast precursors, leading to enhanced osteoblast differentiation and bone formation (16, 17). In single cultures of preosteoblast cells, dihydrotestosterone addition induced the expression of AR protein and activated AR signaling, as measured by AR luciferase activity. This activation of AR signaling in preosteoblast cells was accompanied by evidence of activated canonical Wnt signaling and increased differentiation, as measured by ALP production. Moreover, addition of Wnt inhibitors, DKK-1 and sFRP-1, abrogated the effects of dihydrotestosterone both on Wnt signaling and ALP production in the preosteoblast cells. In contrast, dihydrotestosterone addition to single cultures of MDA-PCa-2b cells had no demonstrable effects on either AR expression or Wnt signaling and resulted in a modest enhancement of cellular proliferation. In contrast, the stimulatory effect of preosteoblasts on MDA-PCa-2b cell proliferation in coculture was markedly enhanced by the addition of androgens (dihydrotestosterone). Furthermore, the addition of Wnt inhibitors to the cocultures abrogated the effect of dihydrotestosterone-stimulated preosteoblasts on MDA-PCa-2b proliferation. Our results strongly imply that androgenic activation of Wnt signaling in preosteoblasts increases the secretion of PCa growth-promoting soluble factors in the cocultures.

We provide evidence that the paracrine growth-promoting effects of androgen-stimulated preosteoblasts on MDA-PCa-2b proliferation are mediated by Wnt signaling in the bone cells. This conclusion is supported by the lack of effect of dihydrotestosterone-one-stimulated preosteoblasts on the proliferation of PC-3 or PC-3ML cells in coculture because both of these PCa cell lines secrete high levels of Wnt inhibitors. Interestingly, the MDA-PCa-2b cell line is the only one of these three bone-derived PCa cell lines that produces an osteoblastic bone reaction in vivo (32). We postulate that the high basal levels of both Wnt inhibitors (DKK-1 and sFRP-1) by the PC-3 and PC-3 ML cell lines prevent the effect of dihydrotestosterone-stimulated preosteoblasts on MDA-PCa-2b proliferation. Although many previous reports have shown interactions between the AR and Wnt signaling pathways in PCa cells (13, 34, 35), this is the first report to show such interactions in preosteoblasts. Previous investigators, using prostate and colon cancer cells, as well as neuronal cells, concluded that there is negative cooperativity between AR and Wnt signaling, i.e., AR-mediated repression of \( \beta \)-catenin/Tcf gene activation (19, 36, 37). It is of note, therefore, that our data show a stimulatory effect of androgens and AR on canonical Wnt signaling in bone preosteoblasts.

Our findings are thus consistent with a mechanism underlying the predilection for PCa cells to metastasize to bone and induce osteoblastic activity. Androgens regulate normal prostate development and are required for prostate tumorigenesis. In both development and carcinogenesis, many of the androgenic effects on prostate epithelial cells are elicited via signaling through stromal AR (38–40). We confirmed that PCa growth in the bone is stimulated by androgenic effects on stromal cells and specifically by activation of Wnt signaling in preosteoblasts. This androgen-mediated Wnt activation in preosteoblasts leads to the secretion of...
soluble factors that stimulate the proliferation of a bone-derived human PCa cell line, MDA-PCa-2b. Once these factors are identified, we will determine whether they also enhance non-bone-derived human prostate cancer cell proliferation. This may have important clinical relevance in the management of PCa. Our data predict that treatment with either androgen-ablative or Wnt-inhibitory therapy early in the course of the disease (before bone metastases) may inhibit both the development of bone metastases and the accompanying osteoblast reaction. It is clear from the current literature that targeting cancer cells directly, even with multimodality approaches, often leads to resistance and relapse.

Therapies aimed at the noncancerous niche, i.e., bone and vascular, may produce more long-lasting disease control.

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References

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