Regulation of Global Gene Expression in the Bone Marrow Microenvironment by Androgen: Androgen Ablation Increases Insulin-Like Growth Factor Binding Protein-5 Expression

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BACKGROUND. Prostate cancer frequently metastasizes to bone. Androgen suppression treatment is initially highly effective, but eventually results in resistant cancer cells. This study evaluates the effects of androgen suppression on the bone and bone marrow (BM). In particular we questioned whether the androgen therapy could adversely facilitate prostate cancer progression through an increase growth factor secretion by the bone microenvironment.

METHODS. Global gene expression is analyzed on mPEDB DNA microarrays. Insulin-like growth factor binding protein-5 (IGFBP5) is detected by immunohistochemistry in mouse tissues and its regulation measured by qPCR and Western blotting in human BM stromal cells. Effects of extracellular matrix-associated IGFBP5 on human prostate epithelial cells are tested in an MTS cell-growth assay.

RESULTS. Castration increases expression of 159 genes (including 4 secreted cytokines) and suppresses expression of 84 genes. IGFBP5 is most consistently increased and the increase in expression is reversed by testosterone administration. IGFBP5 protein is detected in vivo in osteoblasts, BM stromal cells, and endothelial cells. Primary human stromal cell cultures secrete IGFBP5. In vitro, treatment of immortalized human marrow stromal cells with charcoal-stripped serum increases IGFBP5 mRNA expression, which is reversed by androgen supplementation. IGFBP5 is incorporated into the extracellular matrix. Further, IGFBP5 immobilized on extracellular matrices of stromal cells enhances the growth of immortalized prostate epithelial cells.

CONCLUSIONS. Androgen suppressive therapy increases IGFBP5 in the BM microenvironment and thereby may facilitate the progression of prostate cancer. Prostate 67: 1621–1629, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: bone marrow microenvironment; androgen suppression; IGFBP5; prostate cancer
INTRODUCTION

Androgen suppressive therapy has proven a significant benefit when administered in an adjuvant setting together with radiation therapy for the treatment of prostate cancer [1]. Systemic androgen deprivation kills disseminated prostate cancer cells; however, some cells survive the treatment. Survival under androgen deficient conditions may be an inherent property of certain cancer cells but may also be stimulated by factors in the microenvironment. Since prostate cancer commonly metastasizes to the skeleton [2], the environment consists primarily of bone marrow (BM) stroma and hematopoietic BM. Disseminated prostate cancer cells extravasate from the circulation through BM sinusoids [3]. Prostate specific antigen (PSA)-expressing cells are detectable in BM specimens of 54% of patients at the time of prostatectomy, indicating that cancer cells disseminate early [4]. However, disease progression is often delayed by years, suggesting that disseminated cancer cells can remain in a state of dormancy before renewing their growth. Here we investigate whether a decrease in androgen level affects the BM and whether it generates a permissive microenvironment for the growth of prostate cancer cells.

In the BM microenvironment, the androgen receptor is expressed by BM stromal cells, osteoblasts, endothelial cells, osteocytes, and chondrocytes [5,6]. Androgens increase the thickness of bone, augment the hematocrit and regulate the expansion of B-cells [7,8]. The effects of androgen on hematopoiesis are, to a large extent, mediated indirectly through the androgen receptor activity in BM stromal cells [9]. However, androgen-sensitive factors that regulate hematopoiesis are unknown.

Insulin-like growth factors (IGF) and their binding proteins (IGFBP 1–6) are involved in normal and malignant growth of prostate epithelial cells [10]. While there were conflicting results about the expression of the IGF1 receptor (IGFR) in localized and metastatic prostate cancer in formalin-fixed tissues [11–14], a recent study using frozen tissues clearly demonstrates high IGF expression in localized and metastatic cancer as well as in stromal cells surrounding the tumor [15]. In addition, pre-clinical studies with an inhibitory IGFR antibody (A12) reduces the growth of prostate cancer xenografts [16]. IGFs are abundant growth factors in bone and activation of the IGF pathway may lead to ligand-independent activation of the AR [17–19]. The bioavailability of IGF is regulated by a group of IGF binding proteins (IGFBP 1–6). Androgen regulates the expression of IGFBP 2, 3, 4, and 5 in the prostate [20–22]. In the bone, IGFBP 4 and 5 are the two major binding proteins that modulate the IGF activity [23] and IGFBP5 is sequestered by the bone matrix. IGFBP5 may also act independently of IGF as a growth stimulator for osteoblasts, through binding to a separate receptor on the cell surface [24,25]. In human bone and BM, IGFBP5 is expressed in chondrocytes, osteoblasts and osteocytes. These cell types express androgen receptors [26–29], however only androgen regulation of IGFBP 2, 3, 4 and not of IGFBP5 has been examined in-vitro [30].

In this study we sought to measure the effects of androgen suppressive therapy on the BM environment by transcriptional profiling of castrated and sham operated mice. We observed a predominant increase in gene expression after androgen suppression and in particular of IGFBP5. Subsequent in vitro experimentation confirmed IGFBP5 regulation by androgen in human BM stromal cells and demonstrated the functional relevance of elevated IGFBP5 in the BM microenvironment.

MATERIALS AND METHODS

Mice

Castrated or sham-castrated C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) after surgery at 7 weeks of age. Testosterone or placebo pellets (12.5 mg 60-day slow release, Innovative Research of America, Sarasota, FL) were implanted for 4 weeks. Femoral, tibial and humeral bones were collected from 17-week old (young mice, 10 weeks after castration) and 59-week old (old mice, 52 weeks after castration) mice. The experiment was performed in accordance with an approved Animal Care and Use Committee (IACUC) protocol.

Cells

Primary bone stromal cells HB5, HB6, and HB15 were derived from three individuals with IRB approval and maintained in MEM-alpha medium with 10% FBS (Hyclone, Logan, UT). Human immortalized bone stromal cells, HS27a [42] and prostate epithelial cells, P69 [43] were propagated in RPMI1640 with 10% FBS. Human sarcoma MG63 cells were maintained in DMEM with 10% FBS. Human primary prostate stromal and epithelial cells were cultured as previously described [44].

RNA Isolation and Microarrays

Total RNA was isolated from pulverized bone or cultured cells using TRIZOL® (Invitrogen, Carlsbad, CA) and the RNeasy® kit (Qiagen, Valencia, CA). Microarray hybridization and processing of raw data is described in Ref. [45]. Differentially expressed genes...
were analyzed by hierarchical clustering using Cluster 3.0 [46]. The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI. The accession numbers are GSE5775 for castration versus sham-operation and GSE5776 for testosterone replacement versus placebo.

**Reverse Transcription and Quantitative Real-Time PCR (qPCR)**

cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen). Primers for qPCR spanned across intron–exon junctions and the sequences are listed in Supplementary Table I. qPCR conditions with Platinum® SYBR® Green in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) were: 10 min at 95°C, 40 cycles at 95°C for 15 sec, 30 sec at 60°C, 30 sec at 72°C.

**Immunohistochemical Staining**

Mouse femurs were fixed in 10% buffered formalin at 4°C overnight and decalcified in EDTA (Decal Corp., Tallman, NY). Antigens were retrieved with EDTA, pH8 at 95°C for 8 min. The anti-IGFBP5 antibody (sc-6006, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted 1:100 for incubation in the Vantana autostainer model Discover XT™ (Vantana Medical System, Tuscan, AZ).

**Serum Testosterone Measurement**

Mouse blood samples were collected by cardiac puncture and sent for measurement of serum testosterone to the Center for Reproductive Biology, Washington State University, Pullman, WA. The sensitivity of the measurement was 0.10 ng/ml.

**Preparation of Cell Lysate, Conditioned Medium (CM), and Extracellular Matrix (ECM)**

Cells were lysed in RIPA buffer [47] containing protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Serum-free medium was conditioned for 48 hr and concentrated with an Amicon Ultra-15, 5 kDa Centrifugal Filter (Millipore, Billerica, MA). ECM was prepared as described by Knudsen et al. [48]. ECM on plates was used immediately for growth assays or solubilized in RIPA buffer.

**Immunoprecipitation and Immunoblotting**

Proteins (500 µg) were precipitated with 10 µl anti-IGFBP5 (Catalog #06-110, Chemicon International, Inc., Temecula, CA) overnight and proteinG agarose beads (Sigma, Saint Louis, MO) for 2 hr. Total proteins (40 µg) were analyzed on 12% NuPAGE or 4–12% Bis-Tris Gels (Invitrogen) and transferred to Immobilon-P (Millipore). Membranes were blocked with 5% milk and probed with 1:1,000 anti-IGFBP5. Blots were developed with the Pico Chemiluminescent (Pierce Biotechnology, Inc., Rockford, IL).

**Regulation of IGFBP5 Expression in HS27a**

HS27a cells were cultured in Phenol red-free medium with 10% charcoal-stripped FBS (100 ml of FBS stirred with 15 g of dextran-treated charcoal at 4°C overnight and sterilized). Methyltrienolone (R1881, PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA) was added as a synthetic androgen.

**P69 Proliferation in Response to IGFBP5**

HS27a ECM in 24-well plates was incubated with rIGFBP5 without or with human rIGF1 (Sigma) or mono-biotinyl IGF2 (GroPep Limited, Adelaide, SA, Australia) for 4 hr. After the plates were washed, 40,000 P69 cells were seeded per well in serum free medium containing 10 ng/ml IGF1 or IGF2. MTS assays were performed after 48 hr using the CellTiter 96® AQueous cell proliferation assay kit (Promega, Madison WI). The experiment was repeated four times. Statistical analysis was conducted using ANOVA.

**RESULTS**

**Gene Expression Changes in Mouse Bone and Bone Marrow After Androgen Deprivation**

The regulation of gene expression by androgen suppression in the BM has not been reported. Therefore we undertook a global approach to analyze gene expression changes in mouse BM and bone that occur upon castration. We separated bone and BM from young (17 weeks) and old (59 weeks) mice. In addition, we analyzed castrated mice with and without testosterone supplementation.

Analysis of array data revealed that 243 genes exhibited significant and consistent differential expression in bone and BM of young castrated compared to sham-operated mice. Of these, 159 were up-regulated and 84 were down-regulated in. The effectiveness of the castration procedure was documented by a reduction in serum androgen levels and seminal vesicle size.
Next, gene expression changes in BM and bone from young and old mice were compared (Fig. 1A). Of the up-regulated genes, 25/159 were differentially expressed across all arrays. These experiments clearly demonstrate that androgen suppression affects gene expression in the BM, that most of the responsive genes are increased in expression upon androgen suppression and that the expression changes are similar in young versus old mice. Quantitative real-time PCR (qPCR) was used to confirm the expression changes from array data for 22 genes. The fold changes for IGBP5 mRNA as determined by qPCR in three pairs of mice was $2.7 \pm 0.88$ in young mice and $2.0 \pm 0.17$ in old mice. In the same samples the fold expression change for IGBP4 was $0.86 \pm 0.50$ in young mice and $1.14 \pm 0.41$ in old mice. To identify cell types expressing IGBP5 we used immunohistochemistry. IGBP5 expression in sections of mouse bone and BM was observed primarily in osteoblastic cells lining the bone and in endothelial cells (Fig. 2). While we observed weak diffuse staining in the BM stroma, individual BM cells were difficult to discern by morphologic criteria. Thus, the expression of IGBP5 in BM stromal cells was demonstrated subsequently in cultures of primary BM stromal cells.

To determine whether IGBP5 is expressed in human bone and BM stroma, we measured IGBP5 mRNA expression in cultures of primary human BM stromal cells (Fig. 3A). IGBP5 RNA expression was detected in cells from three separate individuals and in immortalized HS27a BM stromal cells. While IGBP5 was expressed in primary cultures of prostate stromal cells, it was not expressed in prostate epithelial cultures under standard growth conditions. IGBP5 protein was secreted from primary marrow stromal cells (Fig. 3B) and HS27a cells (Fig. 3C) and accumulated in the conditioned medium. In addition, IGBP5 became incorporated into the HS27a ECM (Fig. 3C). To exclude the possibility that the IGBP5 antibody cross-reacts with other IGBP5s or that IGBP5 is derived from fetal calf serum, we used MG63 osteosarcoma cells transfected with an IGBP5 containing plasmid (MG63-BP5). A band of the size expected for IGBP5 was only detected in the MG63-BP5 cells, but not in the parent control cells (Fig. 3B).

To determine whether IGBP5 expression is regulated by androgen in human BM stromal cells, we first confirmed expression of the AR in HS27a cells. Both AR mRNA and protein (Fig. 4A) were detectable in cultures of HS27a cells, although to a lesser amount than in fresh prostate tissue. When HS27 cells were cultured in serum that was depleted of steroid hormones by incubation with surface-activated charcoal (IGFBP5) mRNA increased 25-fold after 3 days and IGBP5 protein increased in parallel (Fig. 4B, C). The increase of IGBP5 mRNA was reversed by addition of physiological concentrations of androgen (Fig. 4D). These results demonstrate that the level of AR expression in Hs27a cells is sufficient to regulate IGBP5 expression.

**IGFBP5 Stimulates the Growth of Immortalized Prostate Epithelial Cells**

In contrast to IGBP5 in conditioned medium, which is growth inhibitory, IGBP5 in ECM was shown to

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**TABLE I. Serum Testosterone and Seminal Vesicle Size**

<table>
<thead>
<tr>
<th>Testosterone (ng/ml)</th>
<th>Seminal vesicle (g)</th>
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<tbody>
<tr>
<td>Sham-operation</td>
<td>0.69 ± 0.52</td>
</tr>
<tr>
<td>Castration</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>T-replacement</td>
<td>13.17 ± 1.94</td>
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*Measurements are the average ± standard deviation of tissue samples from three mice.*

**Expression and Regulation of IGBP5 Expression in Bone and Bone Marrow**

IGFBP5 is highly expressed in bone [31]. IGFBP4 is also expressed by cells in bone and BM stroma and antagonizes the activity of IGBP5. However, in contrast to IGBP5, IGFBP4 mRNA expression did not change after castration. The fold expression change for IGBP5 mRNA as determined by qPCR in three pairs of mice was $2.7 \pm 0.88$ in young mice and $2.0 \pm 0.17$ in old mice. In the same samples the fold expression change for IGBP4 was $0.86 \pm 0.50$ in young mice and $1.14 \pm 0.41$ in old mice. To identify cell types expressing IGBP5 we used immunohistochemistry. IGBP5 expression in sections of mouse bone and BM was observed primarily in osteoblastic cells lining the bone and in endothelial cells (Fig. 2). While we observed weak diffuse staining in the BM stroma, individual BM cells were difficult to discern by morphologic criteria. Thus, the expression of IGBP5 in BM stromal cells was demonstrated subsequently in cultures of primary BM stromal cells.

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promote the growth of fibroblasts [32]. We therefore tested whether IGFBP5 bound to ECM could also stimulate the growth of epithelial cells and used P69 immortalized prostate epithelial cells to evaluate the growth-promoting activity of matrix-bound IGFBP5. P69 cells are immortalized human prostate epithelial cells, deficient in Rb and p53 protein function and are highly responsive to IGF1. ECM from HS27a cells was incubated with recombinant IGFBP5 and IGF1 or IGF2. The unbound proteins were removed and P69 cells were plated on the ECM in serum-free medium (Fig. 5A). Two days later, P69 cell numbers were determined using an MTS assay. Compared to untreated matrix, larger numbers of P69 cells were observed on matrices incubated with IGFBP5 alone. Addition of IGF1 or IGF2 to IGFBP5 further increased cell growth. The effects of IGFBP5, IGF1 and IGF2 on growth induction of P69 cells reached a plateau, suggesting a saturable mechanism (Fig. 5B). While growth increased in a linear fashion up to incubation with 25 ng/ml IGFBP5, there was no further increase with 50 or 100 ng/ml IGFBP5. IGF1 or IGF2 in the absence of exogenous IGFBP5 also stimulated cell growth.

**DISCUSSION**

We identified gene expression changes in bone and BM after castration of C57BL/6 mice. The expression of 159 genes increased and the greatest and most...
A consistent increase was observed for IGFBP5. Immunohistochemical staining indicated that BM stromal cells, osteoblasts and endothelial cells express IGFBP5. In the human immortalized bone stromal cell line HS27a, IGFBP5 gene and protein expression were increased by treatment with charcoal-stripped serum and were inhibited by addition of androgen. In-vitro, IGFBP5 was secreted into the medium and deposited into ECM by primary human BM stromal cells and HS27a cells. When bound to ECM, IGFBP5 increased the growth of P69 immortalized prostate epithelial cells. We conclude from these results that IGFBP5 expression increases after androgen ablation, which may promote the growth of prostate cancer cells in the BM environment.

A limitation in the interpretation of castration induced gene expression changes is that castration alters the levels of several hormones, and not only decreases circulating T levels. Thus, changes in other hormones could be responsible for the regulation of gene expression in the BM. Bone is specifically responsive to estrogens, which are decreased in castrated mice and increased with testosterone supplementation. Castration also increases FSH/LH. The distinction between the effects of androgen and estrogen in-vivo would require blockade with an aromatase inhibitor. While the effects of decreased androgen and estrogen cannot be distinguished in-vivo, in-vitro, IGFBP5 induction by charcoal-stripped medium is suppressed by R1881, which cannot be aromatized. These data suggest that the regulation of IGFBP5 expression is mediated by androgen; however it does not exclude a contribution of estrogen in-vivo.

Androgens are known to augment the thickness of bone and accordingly, the AR is expressed in osteoblasts, osteocytes and at sites of endochondral ossification in proliferating, mature and hypertrophic chondrocytes [5]. In several reports, AR copy numbers range between 150 and 5,000 per cell in cultured human BM stromal cells, which include osteoblastic cells [33,34]. Consistent with these results, we detected expression of AR mRNA and protein in HS27a cells. In vivo androgens regulate cell types that lack detectable AR expression presumably through the BM stroma. Anecdotally, androgens were used to treat anemia and the higher hematocrit in men compared to women is attributed to differences in circulating androgen levels [35]. Androgen, but not estrogen or IGF1, regulates the maturation and expansion of the B-cell compartment [8,36,37]. In castrated animals, stromal cells expressing the AR were able to promote the expansion of B-cells from mice afflicted by testicular feminization (Tfm), which possess non-functional AR. In the reverse situation, stromal cells from Tfm mice did not cause changes in B-cell numbers after castration [9]. Interestingly, the activity from the stroma is specific for B-cells and does not affect the T-cell compartment in the BM [38].

Several other studies analyzed the response of IGFBP5 expression to androgen stimulation or androgen suppression and the results are inconsistent. The reason for the discrepancies lies in the difference in cell type and source (cell cultures, xenografts, patient tissue samples), and in the reagents and methods that were used for the analysis. In the prostate and bone, the expression of IGFBP5 RNA is observed in the mesenchymal cells, for example, prostate stromal cells, BM stromal cells and osteoblasts (Ref. [14] and Fig. 3).
Our primary prostate epithelial cultures, which are of the basal/intermediate cell types, did not express IGFBP5 RNA. An immunohistochemical study found about 4% of prostate epithelial cells were stained positive for IGFBP5 protein after 18–43 days of androgen suppressive therapy, an increase compared to the 0.2% as in the placebo group [39]. The regulation of IGFBP5 expression by androgen was also analyzed in xenografts. While IGFBP5 expression increased after castration in the Shinogii xenograft [40], it increased with androgen stimulation in the CWR22 xenograft [41]. This study is the first to examine the regulation of IGFBP5 expression by androgen in bone and BM stromal cells. The results of this study are consistent between in-vivo and in-vitro systems and between mouse and human. Androgen withdrawal clearly increased IGFBP5 expression and the increase in bone and BM in-vivo is sustained for at least 1 year (Fig. 1).

Both, the IGF1 receptor and IGFBP5 are targeted by novel drugs that are undergoing clinical trials for treatment of metastatic prostate cancer [16,40]. This study suggests that the drug target, IGFBP5, is expressed in the microenvironment of metastatic cancer cells. This may increase the opportunity of

**Fig. 4.** Regulation of IGFBP5 expression in HS27a cells by androgen. **A:** AR mRNA expression (left panel) and protein expression (right panel) in HS27a. Lane 1: HS27a; lane 2: prostate tissue, lane 3: negative control. AR protein is detected by Western blot in whole cell lysates of HS27a cells or prostate tissue. B: Induction of IGFBP5 expression by charcoal stripped serum. HS27a cells are cultured in charcoal-stripped serum for indicated time periods. The mRNA expression of IGFBP5 is measured by qPCR and compared to cells grown in regular serum. Expression is normalized to β-actin. C: HS27a cells are cultured in charcoal stripped serum (CS) or regular serum for 2 and 4 days. IGFBP5 expression in equal amounts of whole cell lysate is measured by Western blotting. D: Suppression of IGFBP5 expression by androgen. HS27a cells are cultured in regular serum or charcoal-stripped serum with R1881 testosterone for 24 hr. The experiment was repeated twice with similar results. The fold difference of IGFBP5 RNA expression is calculated as described in B.

**Fig. 5.** Growth stimulation of P69 cells by IGFBP5. **A:** Cartoon of the experimental design. Extracellular matrix (ECM) is prepared from HS27a cells and treated with increasing amounts of recombinant IGFBP5 (BP-5) and 10 ng/ml IGF1 or IGF2. After washing, P69 cells are added together with IGF1 or IGF2 and cell numbers are measured 2 days later. **B:** Growth P69 cells on HS27a matrix. The increase in P69 cell numbers on treated matrixes is calculated relative to growth on untreated matrix. Values represent the average increase in cell numbers from four experiments ± standard deviation. The ANOVA test indicates that addition of IGFBP5 significantly increases P69 cell growth (P < 0.001) and that IGF1 or IGF2 further increase cell growth (P < 0.001). The experiment was repeated twice with different passage numbers of HS27a cells.
IGFBP5 targeted therapies of treating bone metastatic disease. Because IGF1 and IGFBP5 stimulate the progression of cancer cells to androgen independence, early administration of drugs that inhibit their activities may augment the clinical response to androgen ablative treatment. In addition to its therapeutic interest, IGF1 and IGFBP5 levels in the bone and BM could affect the progression of micrometastatic disease. Studies are under way to determine whether progression of micrometastatic disease at the time of radical prostatectomy is increased in men with low serum testosterone levels due to an elevated IGFBP5 concentration. In summary, IGFBP5 functions as a key androgen-sensitive modulator of the BM microenvironment.

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