Inhibition of Constitutively Activated Stat3 Signaling Pathway Suppresses Growth of Prostate Cancer Cells

Zuyao Ni, Wei Lou, Eddy S. Leman, and Allen C. Gao

Abstract

Overexpression of interleukin 6, a downstream target of the GBX2 homeobox gene, has been linked to the progression of prostate cancer. The Janus kinase-signal transducers and activators of transcription signaling pathway is critical for regulating the growth of prostatic cancer cells. The developmental history and characteristic of each of these sublines have been described previously (2). A series of sublines (i.e., G, AT1, AT2, AT6.1, AT6.3, AT3.1, and Mat-LyLu) have been developed from androgen-responsive, slow-growing, nonmetastatic, well-differentiated Dunning R-3327H rat prostatic cancer. The developmental history and characteristic of each of these sublines have been described previously (14, 15). All of the sublines were grown in standard RPMI 1640 (M.A. Bioproducts, Walkerville, MD) containing 10% FCS (Hyclone, Logan, UT), 1 m M glutamine, streptomycin (100 m M/ml), penicillin (100 units/ml), M.A. Bioproducts), and 250 mM dexamethasone (Sigma Chemical Co., St. Louis, MO). The cells were grown at 37°C in 5% CO2 and 95% air.

Cell Culture. Human prostate cancer cell lines (LNCaP, DU145, PC3, and TSU) were maintained in RPMI 1640 supplemented with 10% FCS, as described previously (2). A series of sublines (i.e., G, AT1, AT2, AT6.1, AT6.3, AT3.1, and Mat-LyLu) have been developed from androgen-responsive, slow-growing, nonmetastatic, well-differentiated Dunning R-3327H rat prostatic cancer. The developmental history and characteristic of each of these sublines have been described previously (14, 15). All of the sublines were grown in standard RPMI 1640 (M.A. Bioproducts, Walkerville, MD) containing 10% FCS (HyClone, Logan, UT), 1 mM glutamine, streptomycin (100 μg/ml), penicillin (100 units/ml), M.A. Bioproducts), and 250 mM dexamethasone (Sigma Chemical Co., St. Louis, MO). The cells were grown at 37°C in 5% CO2 and 95% air.

EMSA and Supershift Assay. Whole cell extracts were prepared by lysing cells in a high-salt buffer (20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na3P04, 1 mM Na2VO4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min. The freeze and thaw procedures were repeated again for a total of two times. The supernatant was then centrifuged and harvested. Protein concentrations were determined by using the Coomassie plus protein assay kit (Pierce) according to the manufacturer’s protocol. Whole cell extracts (20 μg) were incubated in a final volume of 20 μl of 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, and 100 μg/ml poly(deoxyinosinic-deoxyctydilic acid) by EMSA with radiolabeled double-stranded Stat3 consensus binding motif 5′-GATCTTCTGG-GAATTCTTAGATC (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min at room temperature. For supershift analyses, the cell extracts were preincubated with antibodies specifically against Stat3α, Stat3β, and Stat1, respectively (Santa Cruz Biotechnology). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed.

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2 To whom requests for reprints should be addressed, at University of Pittsburgh Cancer Institute, BSTW1055, 200 Lothrop Street, Pittsburgh, PA 15213. Phone: (412) 624-0353; Fax: (412) 624-7737; E-mail: gaoac@msx.upmc.edu.

3 The abbreviations used are: IL-6, interleukin 6; JAK, Janus kinase; STAT, signal transducers and activators of transcription; EMSA, electromobility shift assay; MAP, mitogen-activated protein.
STAT3 ACTIVATION IN PROSTATE CANCER

**Transfections.** TSU cells were transfected with dominant-negative Stat3 and vector alone as a control using LipofectAMINE (Life Technologies, Inc.) as described previously (16). The dominant-negative Stat3 and vector-alone expression constructs were kindly provided by Dr. Koichi Nakajima (Osaka University Medical School, Osaka, Japan).

**Growth Assay.** For growth assays, 1 × 10⁶ cells from each clone were plated into 24-well plates in triplicate in RPMI 1640 with 10% FCS. Cells were downshifted to 1% FCS after 48 h. Cell growth was determined by using erythrosin B dye exclusion as described previously (17).

**In Vivo Growth Assay.** Male 4–6-week-old athymic nude mice received s.c. injection in the flank of 2 × 10⁶ cells from parental TSU, vector control (neo), and selected clones transfected with the dominant-negative Stat3 constructs, respectively. The tumor-bearing animals were sacrificed 44 days after inoculation, and the tumors were removed and weighed.

**Statistical Analysis.** Statistical analyses were performed by Student’s t test using paired two sample for means, and P < 0.05 was considered significant.

**Results**

**Stat3 Is Constitutively Activated, and the Activation of Stat3 Is Correlated with Malignant Potential in Prostate Cancer Cells.** We first examined whether Stat3 is activated in prostate cancer cells. Four of the most widely used human prostatic cancer cell lines (i.e., LNCaP, PC3, DU145, and TSU) were tested for activation of Stat3, a critical member of the STAT family, by EMSA with radiolabeled double-stranded Stat3 consensus binding motif. The Stat3 DNA binding activities were detected at variable levels among these human prostate cancer cell lines (Fig. 1A). The strongest Stat3 DNA binding activity was observed in the TSU cell line, the most aggressive of the four human prostate cancer cell lines studied here when grown as xenographs in vivo and the most invasive of the four human prostate cancer cell lines studied here when tested in vitro (2). The weakest Stat3 DNA binding activity was found in the LNCaP cell line, the least aggressive of the four human prostate cancer cell lines studied here (Fig. 1B). The weakest Stat3 DNA binding activity was detected in all of the Dunning sublines (Fig. 1C). The highest Stat3 DNA binding activity was observed in the Mat-LyLu cell line, whereas the lowest Stat3 DNA binding activity was observed in the G cell subline. In terms of metastatic ability, Mat-LyLu is the most aggressive of these cell lines, and G is the least aggressive of these Dunning rat sublines. These results suggest that Stat3 DNA binding activities are correlated with the metastatic abilities in the Dunning rat prostate cancer cells.

**To verify whether the activated Stat3 complexes contained Stat3 or Stat1, supershift analyses using antibodies specifically against Stat3α, Stat3β, and Stat1 were performed as indicated (Fig. 1B).** These results demonstrated that the Stat3, but not Stat1, is constitutively activated in prostate cancer cells.

**Blockade of the Activation of Stat3 by Ectopic Expression of Dominant-negative Stat3 Suppresses the Growth of Prostate Cancer Cells.** The constitutively activated Stat3 found in both human and rat prostate cancer cells and the correlation between the levels of Stat3 DNA binding activity and malignant potential suggest that the JAK-STAT signaling pathway is involved in the progression of prostate cancer. To directly test the role of activation of Stat3 signaling in prostate cancer cells, a dominant-negative Stat3 construct was introduced into the Stat3 constitutively activated TSU cells. The dominant-negative Stat3 construct carries a phenylalanine substitution of the tyrosine residue at 705 that causes a reduction of the tyrosine phosphorylation of wild-type Stat3 and inhibits the action of endogenous Stat3 (18, 19). Stable transfectants containing the dominant-negative Stat3 and vector controls were selected in the presence of G418, subcloned, and tested for Stat3 DNA binding activities by EMSA. As shown in Fig. 2, the endogenously activated Stat3 from two of the representative transfectants with dominant-negative Stat3 (i.e., 3F-11 and 3F-25) was significantly inhibited compared to the parental TSU and vector control.

**To test the effect of inactivation of Stat3 on the growth of TSU cells in vitro, 3F-11, 3F-25 and parental TSU cells and vector control were cultured under identical conditions, and the cell proliferation was examined.** The cell growth of the 3F-11 and 3F-25 clones was significantly suppressed compared to that of the parental TSU cells and the vector control (Fig. 3).

**To test whether the blockade of Stat3 activation has any effect on the in vivo growth of TSU human prostate cancer cells, 2 × 10⁶ cells from the two dominant-negative Stat3 transfectant clones (3F-11 and 3F-25), parental TSU cells, and the vector control, respectively, were...**
injected s.c. into nude mice. As shown in Table 1, TSU parental cells and neo control cells formed tumors in every injection site. In contrast, both dominant-negative Stat3 clones were significantly suppressed \((P < 0.05)\) in their tumorigenicity. Clone 3F-11 cells did not develop any tumors within 44 days of inoculation. Clone 3F-25 developed tumors that were about 30% of the weight compared to the parental TSU cells.

### Discussion

In the present study, we provide evidence that Stat3 activation plays an important role in regulating prostate cancer cell growth and progression. We demonstrate that cells derived from both rat and human prostate cancers have constitutively activated Stat3, with Stat3 activation being correlated with malignant potential. Blockade of activated Stat3 by ectopic expression of a dominant-negative Stat3 in human prostate cancer cells significantly suppresses their growth *in vitro* and their tumorigenicity *in vivo*. In addition, the JAK kinase inhibitor, tyrphostin AG490, inhibited activation of Stat3 and significantly suppressed the growth of human prostate cancer cells. The fact that growth of the human prostate cancer cells was significantly suppressed through inhibition of Stat3 activation by both ectopically expressing a dominant-negative Stat3 and using tyrosine kinase inhibitor tyrphostin AG490 suggests that blockade of the JAK-STAT signaling pathway provides a novel therapeutic approach for prostate cancer.

Whereas significant progress has been made in defining the molecular mechanisms of prostate cancer progression, the specific molecular regulatory pathways affected by these changes have not been fully characterized. Qiu *et al.* (22) demonstrated that IL-6 ligand/IL-6 receptor complex transmits its signals through ErbB2 to a MAP kinase.

### Table 1  In vivo growth of parental TSU, neo, and the dominant-negative Stat3 clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor weight (g) at excision(^a)</th>
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<tbody>
<tr>
<td>TSU</td>
<td>1.2 ± 0.3 (6)(^b)</td>
</tr>
<tr>
<td>Neo</td>
<td>1.0 ± 0.4 (5)</td>
</tr>
<tr>
<td>3F-11</td>
<td>0 ± 0 (6)(^c)</td>
</tr>
<tr>
<td>3F-25</td>
<td>0.38 ± 0.1 (6)(^c)</td>
</tr>
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\(^a\) Mean ± SE  
\(^b\) Numbers in parentheses, number of injection sites/group.  
\(^c\) \(P < 0.05\) compared to the mean of the neo control.

**Fig. 2.** Characterization of the dominant-negative Stat3 clones. Stat3 DNA binding activities in the dominant-negative Stat3 transfectants (i.e., 3F-11 and 3F-25), vector control (neo), and parental TSU cells were examined by EMSA.

**Fig. 3.** Cell growth of the dominant-negative Stat3 transfectants (i.e., 3F-11 and 3F-25), vector control (Neo), and parental TSU cells. Cells \((2 \times 10^5)\) were plated in 6-well plates in triplicate in RPMI 1640 medium with 10% FCS. After 24 h, the cells were downshifted to RPMI 1640 with 1% FCS. The cell number was counted by using erythrosin B dye exclusion. *Error bars*, SD of triplicate samples.

**Fig. 4.** Tyrphostin AG490 inhibits constitutive Stat3 activation and growth of TSU cells. \(A\), cells were incubated for 48 h in culture medium containing the indicated amounts of tyrphostin AG490. Stat3 DNA binding activities were determined by EMSA. \(B\), cells were cultured in medium containing the indicated amounts of AG490 for 48 h, and the cell numbers were counted by using erythrosin B dye exclusion. *Error bars*, SD of triplicate samples.
pathway in prostate cancer cells by inducing tyrosine phosphorylation of ErbB2. Yeh et al. (23) further demonstrated that HER2/Neu increases growth rate, prostate-specific antigen levels, and androgen receptor activation in prostate cancer cells via cross-talk between the MAP kinase and the androgen-androgen receptor signaling pathways. Craft et al. (24) then demonstrated that forced overexpression of HER2/Neu can convert the androgen-dependent prostate cancer cells into androgen-independent prostate cancer cells. In addition, activation of MAP kinase kinase kinase 1 stimulates the transcriptional activity of the androgen receptor signaling pathway independence of the ligand (25). Chen et al. (26) demonstrated that the signal pathway activated by IL-6 is synergistic with epidermal growth factor in human prostate cancer cells.

Our data demonstrate that Stat3 is constitutively activated in both human and rat prostate cancer cells. The Stat3 DNA binding activity is correlated with malignant ability in both human prostate cancer cell lines and a large series of rat Dunning prostate cancer cell lines. TSU and Mat-LyLu, which are the most aggressive cell lines in human and Dunning rat prostate cancer cell systems, respectively, were found to have the highest Stat3 DNA binding activities, whereas LNCaP and G, the least aggressive cell lines in the human and Dunning rat prostate cancer cell systems, respectively, were found to have the lowest Stat3 DNA binding activities. The correlation of Stat3 activation with prostate cancer cell malignancy suggests that the JAK-STAT signaling pathway is commonly activated in human prostate cancer cells and that constitutive activation of this signaling pathway is involved in regulating prostate cancer cell growth and progression.

The factors that induce Stat3 activation in prostate cancer cells are not yet known. We have previously demonstrated that the homeobox gene GBX2 is consistently overexpressed in prostate cancer cells compared to normal prostate epithelial cells and that down-regulation of GBX2 expression inhibits the clonogenic ability and tumorigenicity of prostate cancer cells (2, 3). Homeobox genes including GBX2 encode transcription factors of the helix-turn-helix motif that recognize and bind to specific DNA sequences (27, 28). Through this binding, the homeobox genes either positively or negatively regulate the expression of target genes. We subsequently identified IL-6 as an important downstream target of GBX2 and as one of the mediators of the molecular regulatory pathway initiated by GBX2 that stimulates the growth of prostate cancer cells (4, 5). The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients (29, 30). IL-6 expression is elevated in the sera of patients with metastatic prostatic carcinoma (31). We further demonstrated that ectopic expression of IL-6 stimulates prostate cancer cell growth accompanied by activation of the Stat3 signaling pathway (5). Here we demonstrate that activation of the Stat3 signaling pathway plays an important role in regulating prostate cancer cell growth and progression. Taken together, we propose that activation of Stat3, a critical mediator for prostate cancer cell growth, occurs partly through transcriptional activation of IL-6 by overexpression of the GBX2 homeobox gene in prostate cancer cells.

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References

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