Inhibition of Constitutively Activated Stat3 Signaling Pathway Suppresses Growth of Prostate Cancer Cells¹

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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 transmits interleukin 6-mediated signals from cell surface receptors to the target genes in the nucleus and is critical in mediating cellular growth and differentiation. 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. Furthermore, the Janus kinase inhibitor, tyrphostin AG490, inhibited the constitutive activation of Stat3 and suppressed the growth of human prostate cancer cells. These results indicate that activation of Stat3 signaling is essential in the progression of prostate cancer cells and suggest that targeting Stat3 signaling may yield a potential therapeutic intervention for prostate cancer.

Introduction

Prostate cancer is the most frequently diagnosed cancer and is the second leading cause of cancer death in American men (1). 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. We 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). We further identified IL-6 as one of the downstream targets of GBX2 (4). In a recent study, we demonstrated that ectopic expression of IL-6 stimulated prostate cancer cell growth accompanied by activation of the Stat3 signaling pathway (5). These results prompted us to investigate the importance of the Stat3 signaling pathway in human prostate cancer cells.

Cytokines and growth factors play central roles in the regulation of a wide array of cellular functions in eukaryotic cells by affecting target cells through the JAK-STAT pathway (6, 7). Membrane-associated JAK tyrosine kinases are activated by ligand binding, which selectively recruits inactive cytoplasmic transcription factors, STATs, and activates them by phosphorylation. The activated STATs then translocate to the nucleus and activate target gene transcription (6, 7).

Constitutively activated Stat proteins are found in various types of tumors including leukemia, breast, and head and neck tumors (8–10). Activation of Stat3 has been observed in cells transformed in vitro with v-src and abl oncogenes (11, 12). Furthermore, the Stat3 mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3 produced a spontaneously activated Stat3 that induces cellular transformation and tumor formation in nude mice (13). These results suggest that constitutively activated Stat3 participates in the development of malignant cancer. It is important to know whether or not activation of the Stat3 signaling pathway is critical for regulating the growth of prostatic cancer cells.

In this report, we examined the importance of JAK-STAT signaling in prostate cancer cells. We demonstrated 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 or by JAK kinase inhibitor AG490 significantly inhibited the growth of human prostate cancer cells. Our results demonstrate that activation of the Stat3 signaling pathway is critical for the growth of prostate cancer cells and suggest that targeting Stat3 signaling may yield a potential therapeutic intervention for prostate cancer.

Materials and Methods

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 m M glutamine, streptomycin (100 m g/ml), penicillin (100 units/ml), M. A. Bioproducts), and 250 m M 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 Na₃P₂O₇, 1 mM NaVO₄, 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’-GATCTCTTGG-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 Stat3a, 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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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.
**Transfections.** TSU cells were transfectioned with dominant-negative Stat3 and vector alone as a control by 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, $1 \times 10^4$ 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 $2 \times 10^6$ 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 (TSU, PC3, DU145, and LNCaP). These results suggest that Stat3 is commonly activated in human prostate cancer cells and that activation of Stat3 is correlated with malignant potential, at least in these human prostate cancer cell lines.

To test whether the JAK-STAT signaling pathway is also activated in rat prostate cancer cells and whether the activation of the Stat3 is related to metastatic potential, Stat3 DNA binding activities were examined in a large series of Dunning rat prostatic cancer sublines that differ widely in their malignant ability. Activation of Stat3 DNA binding activities was detected in all of the Dunning sublines (Fig. 1A). 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 cell lines.

**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, $2 \times 10^6$ 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 of the parental TSU cells.

Tyrphostin AG490 Blocks Constitutive Stat3 Activation and Inhibits the Growth of Prostate Cancer Cells. We next examined whether a JAK-specific inhibitor, tyrphostin AG490, which has been shown to effectively block Stat3 activation in acute lymphoblastic leukemia and in mycosis fungoides (20, 21), could block the constitutive activation of Stat3 and suppress the growth of TSU cells. As shown in Fig. 4A, tyrphostin AG490 strongly inhibited constitutively activated Stat3 in TSU cells at a concentration of 100 μM. The proliferation of the TSU cells was significantly suppressed by tyrphostin AG490 at a concentration ≥ 12.5 μM, with an IC50 of 25 μM (Fig. 4B).

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

| Cell line | Tumor weight (g) at excision
<table>
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<tr>
<td>TSU</td>
<td>1.2 ± 0.3 (6)</td>
</tr>
<tr>
<td>Neo</td>
<td>1.0 ± 0.4 (5)</td>
</tr>
<tr>
<td>3F-11</td>
<td>0.38 ± 0.1 (6)</td>
</tr>
<tr>
<td>3F-25</td>
<td>0.38 ± 0.1 (6)</td>
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Table 1 In vivo growth of parental TSU, neo, and the dominant-negative Stat3 clones

*Mean ± SE.
Numbers in parentheses, number of injection sites/group.
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 × 10⁵) 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 independently 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 derived directly from patients (29, 30). IL-6 expression is elevated in the sera of patients with metastatic prostate 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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