Constitutive Activation of Stat3 in Human Prostate Tumors and Cell Lines: Direct Inhibition of Stat3 Signaling Induces Apoptosis of Prostate Cancer Cells

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ABSTRACT

Signal transducers and activators of transcription (STATs) were identified originally as key components of cytokine signaling pathways. More recently, constitutive activation of STAT proteins has been detected in a wide variety of human tumor specimens and tumor cell lines. Here, we examined the activation of one STAT family member, Stat3, in human prostate cancer cell lines and primary prostate tumors. An analysis of 45 adenocarcinomas obtained at radical prostatectomy revealed elevated levels of constitutive Stat3 activation in 37 (82%) of 45 of the tumors compared with matched adjacent nontumor prostate tissues. A highly specific immunohistochemical assay for detection of phospho-Stat3 revealed that elevated Stat3 activity was localized primarily in the tumor cells of prostate carcinoma specimens. Furthermore, higher levels of Stat3 activation in patient specimens were correlated significantly with more malignant tumors exhibiting higher Gleason scores. In addition, all of the three human prostate cancer cell lines examined (DU145, PC3, and LNCaP) displayed constitutive activation of Stat3. Substantially lower levels of Stat3 activation were detected in LNCaP cells; however, stimulation with interleukin 6 induced a significant increase in Stat3 DNA-binding activity in these cells. Moreover, the direct inhibition of constitutive Stat3 signaling in DU145 cells using antisense Stat3 oligonucleotides induced growth inhibition and apoptosis. Our findings demonstrate that constitutive activation of Stat3 occurs frequently in primary prostate adenocarcinomas and is critical for the growth and survival of prostate cancer cells. These studies further suggest that Stat3 signaling represents a potentially novel molecular target for prostate cancer therapy.

INTRODUCTION

The natural history of prostate cancer is poorly understood, but progression appears to be related to stage and grade of tumor (reviewed in Refs. 1–4). Biologically, prostate cancer represents a heterogeneous disease entity that exhibits various degrees of aggressiveness, patterns of metastasis, and response to therapy. Therefore, there is a great need to identify molecular prognostic factors that allow stratification of prostate cancer patients into homogeneous treatment groups (4, 5). Furthermore, the identification of new molecular targets may lead to more effective treatments for prostate cancer.

STATs⁴ were identified originally as central players in signaling pathways involved in mediating responses to IFNs and other cytokines (reviewed in Refs. 6–8). On phosphorylation of STAT monomers by tyrosine kinases, the monomers dimerize, translocate to the nucleus, and bind to specific promoter sequences, thereby inducing gene expression. STAT proteins have essential functions in signaling pathways critical to normal cellular processes including immune function, development, differentiation, proliferation, and survival (9–12). One of the cytokines known to activate Stat3 is IL-6. IL-6 regulates a wide variety of biological responses and stimulates or inhibits cellular growth in a cell type-dependent manner (13, 14). Furthermore, activation of Stat3 by IL-6 has a critical role in the pathogenesis of multiple myeloma by preventing apoptosis (15). In addition, IL-6 has been suggested to participate in malignant progression of prostate cancer (16).

Earlier studies have reported that constitutive activation of one STAT family member, Stat3, occurs in fibroblasts stably transformed by the Src oncoprotein and other oncoproteins that activate tyrosine kinase signaling pathways (17–20). More recent studies have demonstrated that Stat3-mediated expression of cellular genes is required for Src-induced oncogenic transformation (21–23). In addition, constitutive STAT activation occurs frequently in a variety of human tumor cell lines and primary human tumors including leukemias, lymphomas, multiple myeloma, head and neck cancer, and breast cancer (24–27). Moreover, a constitutively activated mutant of Stat3 is sufficient to induce some properties of transformed cells, including tumorigenicity (28). Collectively, these findings provide evidence that signaling by Stat3 protein participates in regulating the processes of cell growth and survival during oncogenesis.

Because cytokines including IL-6 have been implicated in prostate cancer, we examined Stat3 signaling in primary tumor specimens from prostate cancer patients and in human prostate cancer cell lines. Using both a biochemical Stat3 DNA-binding assay and an immunohistochemical phospho-Stat3 assay, we demonstrate that Stat3 is constitutively activated with high frequency in prostate adenocarcinomas compared with matched adjacent nontumor prostate tissues. In addition, we used antisense Stat3 oligonucleotides to block Stat3 signaling and to evaluate the biological role of activated Stat3 in model prostate cancer cell lines. Results indicate that activated Stat3 transduces signals that are required for growth and survival of human prostate cancer cells. Our findings implicate constitutive activation of Stat3 in malignant progression of prostate cancer and suggest that Stat3 represents a promising molecular target for novel prostate cancer therapy.

MATERIALS AND METHODS

Patient Identification. In contrast to other solid tumors, prostate cancer is not easily identified by gross inspection. Because of the wide use of PSA screening and early diagnosis, a significant number of patients who undergo radical prostatectomy reveal very little residual tumor. To assure that enough tumoral tissue was collected from the prostatectomy specimens to perform all of the proposed molecular studies, we used only prostatectomy specimens from patients with a prior diagnosis of prostate cancer in at least 50% of two of six prostate biopsies. The ideal patient was the one with two or three positive biopsies in one side and negative biopsies on the contralateral side. The findings on the prostate biopsies, which are routinely performed before pros-
tectomy, were used for patient selection and as guidance for tumoral and nontumoral tissue collection.

**Pathology Evaluation.** Prostatectomy specimens were sent immediately to the pathology laboratory on ice with a label indicating the time of excision (to monitor processing time from excision to snap-freezing). Every effort was made to minimize processing time to less than 15 min because it was extremely important to snap-freeze tumor specimens in liquid nitrogen as soon as possible to preserve the activation state of STAT proteins in tumor cells. The specimen was cut at 5-mm intervals, and areas of normal and tumor tissue were identified grossly with the guidance of biopsy results obtained before prostatectomy. The frozen sections were evaluated by the pathologists (J. D., L. B. M.) to confirm the diagnosis of both normal and tumor tissue. This approach assured reliable comparison of biochemical data in tumoral and nontumoral tissue. Excess tissue not required for pathological examination of the specimen was obtained for the purposes of this study. Informed consents were signed by all patients to allow the use of their tissues in these experiments as part of a clinical protocol approved by the local Institutional Review Board. Tissue adjacent to (mirror-image area) the confirmed tumor or normal tissue by frozen section evaluation was snap-frozen immediately in liquid nitrogen and sent to the Moffitt Cancer Center Tumor Bank for future STAT studies. The remainder of the prostatectomy specimen was fixed in formalin and processed for routine histological examination and immunohistochemical analysis.

**Cell Lines and Culture Conditions.** The human prostate carcinoma cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS. IL-6 stimulation experiments were performed using 10 ng/ml IL-6 in serum-free medium for the indicated times.

**Nuclear Extract Preparation and EMSA.** For STAT analysis by EMSA, the specimens were placed in a prechilled pestle and mortar on liquid nitrogen, and the tissue was pulverized in the frozen state. Nuclear extracts were prepared as described previously (17, 20) from primary tissue specimens with approximately 10^6 cells by high-salt extraction into 30–70 μl buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM Na_4P_2O_7, 1 mM DTT, 0.5 mM phenylenediamine, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain] producing about 30–90 μg of total protein. For EMSA, 5 μg of total extracted protein was used for each lane. EMSA was performed using a 3^2P-labeled oligonucleotide probe containing a hSIE derived from the c-fos gene promoter (sense strand, 5'-AGCTCTTATCCGCCAGCTGCA-3') that binds activated Stat3 and Stat3 proteins (17). Protein-DNA complexes were resolved by nondenaturing PAGE and detected by autoradiography. For controls, STAT activity was determined in matched specimens obtained from nontumor tissues adjacent to the tumor, and also in NIH3T3 cells transformed by the Src oncoprotein (17) as an internal reference standard. Thus, levels of STAT activation were compared in nontumor and tumor tissues of the same patient, and normalization to the Src internal reference standard allowed comparison across different EMSA gels. Quantification of Stat3 activation levels was performed using ImageQuant software analysis of scanned EMSA gel bands. Anti-Stat1 and Stat3 polyclonal antibodies (Santa Cruz Biotechnol) were used to identify specific STAT family members. For use in supershift assays, 1 μl of the STAT antibodies was incubated with nuclear extracts for 20 min at room temperature before the addition of radiolabeled probe and electrophoresis.

**Oligonucleotides and Transfections.** The Stat3 antisense (5'-GCT CCA CTA TCT GCT GCT TC-3') or control mismatch oligonucleotides (5'-GCT CCA ATA CCC GTT GCT TC-3') were synthesized using phosphorothioate chemistry. To increase stability, oligonucleotides were synthesized with 2^-methoxyethyl modification of the five terminal nucleotides (underlined; Ref. 29). Transfections were carried out by the Lipofectamine-Plus method as described by the supplier (Life Technologies, Inc.). Briefly, DU145 cells were seeded at 2 × 10^5 cells/10-cm tissue-culture plates 18 h before transfection in RPMI 1640 supplemented with 10% FBS. Immediately before transfection, cells were washed once with PBS. Cells were transfected for 3 h with Lipofectamine-Plus (LF+), alone, with LF+/Stat3 antisense oligonucleotides, LF+/Stat3 mismatch oligonucleotides, or not transfected. The final concentration of the oligonucleotides was 250 nM. The transfection was terminated by aspirating the transfection medium, washing the cells one time with PBS and adding fresh RPMI 1640 containing 10% FBS. Another 24 h later, the non-adherent cells were washed off and the remaining cells were lysed EMSA or Western blot analysis.

**Antisense and Proliferation Assays.** For apoptosis assays, DU145 cells were treated for 24 h with Lipofectamine-Plus alone or with Stat3 antisense or Stat3 mismatch control oligonucleotides. Treated cells were stained with an antibody specific for activated caspase-3 using the supplier’s protocol (Becton Dickinson PharMingen) and analyzed by flow cytometry. For proliferation assays, cells numbers were determined by counting with a hemocytometer using trypsin blue exclusion.

**Immunohistochemical Detection of Phospho-Stat3.** Cytophins from LNCaP, DU145, and PC3 cells lines without treatment, LNCaP cells stimulated with IL-6 (10 ng/ml), and DU145 cells transfected with Lipofectamine-Plus (LF+) alone, with LF+/Stat3 antisense oligonucleotides, or LF+/Stat3 mismatch oligonucleotides were fixed in 95% ethanol for 10 min. Forty-five pairs of matched primary prostate tumors with normal tissue were fixed in 10% neutral-buffered formalin and embedded in serial 3–4-μm paraffin blocks. Five-μm thick sections were stained with H&E for histological examination. Representative histological sections and cytophins were then immunostained to localize Stat3. Immunostaining for phospho-Stat3 was performed using a rabbit antihuman polyclonal antibody (Phospho-Tyr705-Stat3; Cell Signaling, Beverly, MA). As negative controls, rabbit immunoglobulins (Vector, Burlingame, CA) were used to replace primary antibody. The 5-μm thick tissue sections described above were deparaffinized and hydrated in deionized water. The immunohistochemical staining was performed manually at room temperature, using the avidin-biotin-peroxidase complex method (Vectorstain Elite ABC kit; Vector Lab). Briefly, pretreatment for antigen retrieval with a pressure cooker involved heating cytophins and tissue sections with a microwave oven, in 250 ml of unmasking solution (Vector Lab) for 20 min at high-power level, followed by 20 min of cooling time. Slides were then treated with 0.025% trypsin in 50 mM Tris (pH 7.6) for 5 min at 37°C without prewarming. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 3% hydrogen peroxide for 10 min. After washing with PBS for 5 min, slides were blocked with normal serum and 3% BSA for 10 min, followed by incubation with the phospho-Stat3 primary antibody, at a dilution of 1:400, overnight at 4°C. After rinsing with PBS for 5 min, slides were incubated with a biotinylated secondary antibody for 60 min and washed again. After washing with PBS for 5 min, slides were incubated with avidin-biotin complex for 1 h and washed again. Chromogen was developed with 3,3-diaminobenzidine (DAB Substrate kit for peroxidase; Vector Lab) or Nova-red (Nova-red Substrate kit for peroxidase; Vector Lab). All of the slides were lightly counterstained with hematoxylin for 30 s before dehydration and mounting. The pathologists (L. B. M., N. A.) evaluated immunohistochemical reactions in the cell lines and primary tumors.

**Image Analysis Cytometry.** The computer-assisted CAS-200 Image Analysis System was used to quantify immunohistochemical staining of positive nuclei/total number of cells (×100 for percent). A minimum of 300 nonoverlapping and well-preserved cells were measured within at least 10 adjacent ×400 magnification fields in each sample. Quantification of nuclei positive for phospho-Stat3 staining was assessed in tumor and normal prostatic gland cells. The CAS-200 system operator was unaware of the Stat3 DNA-binding results by EMSA at the time of evaluation. Data were analyzed for statistical significance using the Wilcoxon signed-rank test.

**RESULTS**

**Elevated Stat3 DNA-binding Activity in Human Primary Prostate Tumors.** To determine whether constitutive activation of Stat3 is associated with prostate cancer in vivo, we performed EMSA analysis using nuclear extracts prepared from a series of 45 primary prostate tumor (T) specimens with matched adjacent nontumor (N) prostate tissues. As a positive control (+C) and internal reference standard for comparison across EMSA gels, identical aliquots of nuclear extracts were used from NIH3T3 cells transformed by the Src oncoprotein that have been shown to contain high levels of Stat3 activation (17–20). Quantitative pairwise comparative analysis of EMSA results (Fig. 1) revealed elevated Stat3 DNA-binding activity, to different extents, in 82% (37 of 45) of primary adenocarcinoma specimens examined (Lanes T1 through T45) relative to matched adjacent nontumor tissues.
Elevated Stat3 DNA-binding activity in human primary prostate tumors. EMSA analysis was performed using nuclear extracts prepared from 45 representative sets of matched primary adenocarcinoma and adjacent non-tumor tissue specimens (T, primary tumors; N, matched non-tumor tissues adjacent to primary tumors) using the radiolabeled hSIE probe, which binds activated Stat3 with high affinity. Results demonstrate elevated Stat3 activities in 37 of 45 tumor specimens compared pairwise with matched non-tumor tissues. The positive control (+C) consisted of NIH3T3 cells transformed by the Src oncoprotein with high levels of constitutive Stat3 activation, which provided an internal reference standard for comparison across EMSA gels. This is the only case (T5) in which Stat3 activity was not detectable in tumor tissue. It is also notable that 29% (13 of 45) of adjacent non-tumor tissues exhibited Stat3 DNA-binding activity higher than basal levels present in a majority of non-tumor prostate tissues. Only one case displayed no detectable Stat3 activity in the tumor tissue (Lane T5).

A high frequency of constitutive Stat3 activation is associated with primary prostate tumors in vivo, consistent with a role for activated Stat3 signaling in prostate cancer.

Analysis of Stat3 DNA-binding Activity in Human Prostate Cancer Cell Lines. To evaluate Stat3 activation in model prostate cancer cell lines, we performed EMSA analysis with the three well-characterized human prostate cancer cell lines DU145, PC3, and LNCaP. As shown in Fig. 2A, Stat3 DNA-binding activity is present in nuclear extracts prepared from all three of the prostate cancer cell lines examined. DU145 cells have the highest level, whereas LNCaP cells have the lowest level and are the least malignant, in agreement with the earlier findings of Ni et al. Antibodies to Stat3, but not Stat1, could block and supershift this DNA-binding activity, confirming that the activity corresponds to Stat3 in all three cell lines (data not shown). We examined whether IL-6 could induce further Stat3 DNA-binding activity in the LNCaP cells. Treatment of serum-starved LNCaP cells with 10 ng/ml IL-6 induced a time-dependent activation of Stat3 (Fig. 2B). Antibodies to Stat3, but not Stat1, blocked this DNA-binding activity, confirming that it corresponds to Stat3. Consistent with the cell line data, antibody supershift analysis of all 45 patient tumor specimens from Fig. 1 demonstrated that the DNA-binding activity corresponds to Stat3 and not Stat1 (Fig. 2C, and data not shown).

Detection of Activated Phospho-Stat3 by Immunostaining of Human Prostate Cancer Cell Lines and Primary Tumors. To optimize the immunohistochemical protocol, we performed immunostaining with phospho-Stat3 antibody on cytospins with the model prostate cancer cell lines DU145, PC3, and LNCaP. This antibody reacts with the phosphorylated tyrosine 705 residue that is indicative of Stat3 activation (7, 8). As shown in Fig. 3A, phospho-Stat3 is detected in the nucleus with different intensities in all three of the human prostate cancer cell lines examined. The intensity of red stain (Nova-Red) corresponds to the level of active phospho-Stat3, whereas

**Fig. 1.** Elevated Stat3 DNA-binding activity in human primary prostate tumors. EMSA analysis was performed using nuclear extracts prepared from 45 representative sets of matched primary adenocarcinoma and adjacent non-tumor tissue specimens (T, primary tumors; N, matched non-tumor tissues adjacent to primary tumors) using the radiolabeled hSIE probe, which binds activated Stat3 with high affinity. Results demonstrate elevated Stat3 activities in 37 of 45 tumor specimens compared pairwise with matched non-tumor tissues. The positive control (+C) consisted of NIH3T3 cells transformed by the Src oncoprotein with high levels of constitutive Stat3 activation, which provided an internal reference standard for comparison across EMSA gels. This is the only case (T5) in which Stat3 activity was not detectable in tumor tissue. It is also notable that 29% (13 of 45) of adjacent non-tumor tissues exhibited Stat3 DNA-binding activity higher than basal levels present in a majority of non-tumor prostate tissues. Only one case displayed no detectable Stat3 activity in the tumor tissue (Lane T5).

**Fig. 2.** Constitutive activation of Stat3 in model human prostate carcinoma cell lines. A, EMSA analysis of Stat3 DNA-binding activity in nuclear extracts prepared from human prostatic cancer cell lines using the radiolabeled hSIE probe. LNCaP is the least malignant cell line, and it was found to have the lowest Stat3 DNA-binding activity. NIH3T3 cells transformed by the Src oncoprotein serve as a positive control for constitutive Stat3 activation. B, kinetics of Stat3 activation by IL-6 in LNCaP cells. Cells were serum-starved to reduce the basal levels of Stat3 activation before stimulation with 10 ng/ml IL-6 for the indicated times (0 min, 10 min, 20 min, 30 min, 40 min, 90 min). Stat3 DNA-binding activity was positively confirmed by supershift analysis with antibodies to Stat3 (Lane 8) but not Stat1 (Lane 7). C, supershift analysis of representative primary tumor specimens from Fig. 1 demonstrates that the DNA-binding activities correspond to Stat3 and not Stat1. Similar results were obtained with all 45 of the patient tumor tissue specimens (data not shown).
the blue stain (hematoxylin) is the counterstain. The levels of phospho-Stat3 immunoreactivity correlate well with the levels of Stat3 DNA-binding activity as measured by EMSA in these cells (compare Figs. 2A and 3A), with DU145 cells having the highest and LNCaP cells having the lowest levels.

The specificity of the immunoreaction for phospho-Stat3 was confirmed by specific ablation of Stat3 protein expression using antisense Stat3 oligonucleotides (see Fig. 4 below). After optimization of the immunohistochemical protocol, we used the phospho-Stat3 antibody to investigate the levels and cytological locations of activated Stat3 in the tumor specimens from prostate carcinoma patients. Immunohistochemistry demonstrated that low levels of nuclear phosphorylated Stat3 are restricted to the basal epithelial cells in normal (N) tissue adjacent to tumor tissue (Fig. 3B). In contrast, elevated levels of phospho-Stat3 are detected primarily in the nuclei of epithelial tumor cells in tumor (T) specimens (Fig. 3B). To quantify these observations, epithelial cells within the glands of prostate samples were analyzed by computer-assisted image analysis and the data are summarized in Fig. 3C. Statistically, the difference in phospho-Stat3 levels between the malignant specimens and nontumor specimens was highly significant (P < 0.001) using the Wilcoxon signed-rank test. Increased Stat3 tyrosine phosphorylation in the tumor cells detected by immunohistochemistry correlates well with elevated Stat3-DNA binding activities as detected by EMSA for these specimens (Fig. 1, and data not shown).

**Elevated Levels of Phospho-Stat3 Correlate with High Gleason Score in Prostate Tumor Specimens.** The levels of activated phospho-Stat3 protein in prostate tumors as detected by immunohistochemistry were compared with the clinicopathological characteristics of 45 prostate cancer patients. As summarized in Table 1, increased phospho-Stat3 levels were highly correlated (P < 0.007) with more malignant tumor specimens exhibiting Gleason scores ≥7. By contrast, there was no significant correlation between levels of phospho-Stat3 and either initial serum PSA levels or clinical stage (Table 1). It was not possible to assess the correlation between levels of phospho-Stat3 and established parameters of disease progression (e.g., PSA levels after prostatectomy) because of the short time of follow-up after treatment of this cohort of patients. Thus, higher phospho-Stat3 levels correlate well with
these results demonstrate that the antibody and expected (Fig. 4). In addition, stimulation of LNCaP cells antisense oligonucleotide demonstrated that the phospho-Stat3 staining of cytospins prepared from DU145 cells treated with Stat3 antisense oligonucleotide (Fig. 3) is specific for phospho-Stat3, we blocked Stat3 expression diminish Stat3 protein expression. To determine the consequences of down-modulating Stat3 protein expression in the model prostate cells, we transfected DU145 cells with Stat3 antisense oligonucleotide as described previously (31). Twenty-four h after transfection, EMSA was performed to measure levels of constitutive Stat3 DNA-binding activity. Fig. 4A, shows greatly decreased Stat3 DNA-binding activity in the DU145 cells transfected with Stat3 antisense oligo (Lane 3) compared with nontransfected cells (Lane 1), Lipofectamine-Plus (LF+) transfection reagent alone (Lane 2), or Stat3 mismatch control oligonucleotide (Lane 4). Antibodies to Stat3 could block and supershift this DNA-binding activity, confirming that the activity corresponds to Stat3. Western blot analysis of total Stat3 expression showed that treatment of DU145 cells with LF+/Stat3 antisense oligonucleotide alone or Stat3 mismatch control oligonucleotide. Collectively, these findings indicate that activated Stat3 signaling is required for the growth and survival of prostate cancer cells.

**DISCUSSION**

In this study, we investigated whether constitutive activation of Stat3 protein is associated with prostate cancer in vivo. Significantly, EMSA analysis using nuclear extracts prepared from 45 primary prostate adenocarcinomas revealed elevated Stat3 DNA-binding activity in 82% of the tumor specimens compared with matched adjacent nontumor prostate tissues. These findings were confirmed by an independent measure of Stat3 activation based on immunohistochemical detection of phospho-Stat3. This immunohistochemical assay is useful for histological localization and quantification of activated Stat3 in primary tumor specimens. In addition, model human prostate cancer cell lines displayed constitutive activation of Stat3 DNA-binding activity, which could be further induced by IL-6 or blocked by antisense oligonucleotides specific for Stat3. Importantly, direct inhibition of Stat3 signaling was accompanied by growth inhibition and induction of apoptosis in prostate cancer cells.

Several lines of evidence suggest that IL-6 that are produced by tumor cells themselves or by adjacent stromal cells can modulate the growth of prostate tumor cells in an autocrine or paracrine manner (16, 32–34). The expression of IL-6 and its receptor has been detected not only in human prostate cancer cell lines but, more significantly, in human prostate carcinoma and benign prostate hyperplasia (BPH) specimens obtained directly from patients (35, 36). In addition, levels of IL-6 are elevated in the serum of patients with hormone-refractory and metastatic prostate carcinoma (35, 36). These findings suggest that the elevated Stat3 activation levels that we observed in prostate tumor tissues may arise from autocrine and/or paracrine stimulation by IL-6. Furthermore, it is possible that the elevated Stat3 activation detected in some of the adjacent nontumor tissues could also be induced by IL-6. Consistent with this possibility, levels of phospho-Stat3 were generally higher in nontumor tissues that were closer to the regions of tumor than in those that were farther away, suggesting that factors such as IL-6 secreted by tumor cells may induce Stat3 activation in adjacent tissues.5

Our findings are consistent with recent studies showing that IL-6 stimulates prostate cancer cell growth and activation of Stat3 signaling (37) and that the inhibition of Stat3 signaling blocks the growth of prostate cancer cells (30). In contrast, other studies (38, 39) suggested that Stat3 signaling correlated with IL-6-induced growth arrest and differentiation of prostate cancer cells. The apparent discrepancy between the latter (38, 39) and the former studies (30, 37) as well as the present study may be explained by other factors, such as androgens, which could modulate responses to Stat3 signaling. In this context, it may be significant that IL-6 undergoes a transition from being growth inhibitory to being growth stimulatory during progression to hormone-refractory prostate cancer (40). Moreover, recent studies indicate that IL-6 induces androgen receptor-mediated gene regulation through the Stat3 protein (41), raising the possibility that IL-6-induced Stat3 activation may contribute to the development of hormone-refractory prostate cancer. Consistent with a growth-stimulatory role of Stat3 in prostate cancer (30, 37, 41), our results demonstrate that the direct inhibition of Stat3 signaling by antisense Stat3 oligonucleotides blocks the growth and survival of these cells.

Our findings reported here are also in agreement with recent studies showing that phospho-Stat3 levels are elevated in malignant prostate

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**Table 1** Statistical correlation between levels of immunohistochemical phospho-Stat3 staining and clinicopathological characteristics of 45 patients with prostate adenocarcinomas

<table>
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<th>Tumor characteristics</th>
<th>0% n (%)</th>
<th>≤25% n (%)</th>
<th>25–50% n (%)</th>
<th>≥51% n (%)</th>
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<td>0–4</td>
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<td>3 (7)</td>
<td>3 (7)</td>
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more aggressive disease in prostate cancer as determined by Gleason score at the time of radical prostatectomy.

**Antisense Stat3 Oligonucleotides Block Stat3 Protein Expression and DNA-binding Activity in Prostate Cancer Cells.** To determine the consequences of down-modulating Stat3 protein expression in the model prostate cells, we transfected DU145 cells with Stat3 antisense oligonucleotide as described previously (31). Twenty-four h after transfection, EMSA was performed to measure levels of constitutive Stat3 DNA-binding activity. Fig. 4A, shows greatly decreased Stat3 DNA-binding activity in the DU145 cells transfected with Stat3 antisense oligo (Lane 3) compared with nontransfected cells (Lane 1), Lipofectamine-Plus (LF+) transfection reagent alone (Lane 2), or Stat3 mismatch control oligonucleotide (Lane 4). Antibodies to Stat3 could block and supershift this DNA-binding activity, confirming that the activity corresponds to Stat3. Western blot analysis of total Stat3 protein levels (Fig. 4B) demonstrates that Stat3 antisense (Lane 3) but not mismatch oligonucleotides (Lane 2) were able to substantially diminish Stat3 protein expression.

To establish that the immunohistochemical assay described above (Fig. 3) is specific for phospho-Stat3, we blocked Stat3 expression using antisense Stat3 oligonucleotide. This block in Stat3 expression should ablate specifically the phospho-Stat3 antigen and, thus, represents a highly rigorous control for antibody specificity. Immunostaining of cytospins prepared from DU145 cells treated with Stat3 antisense oligonucleotide demonstrated that the phospho-Stat3 staining was abolished (Fig. 4C). In addition, stimulation of LNCaP cells with IL-6 resulted in increased phospho-Stat3 immunostaining, as expected (Fig. 4D). These results demonstrate that the antibody and immunohistochemical methods used are specific for phospho-Stat3 and that the immunostaining results correlate well with Stat3 DNA-binding activities as measured by EMSA analysis.

**Blocking Stat3 Signaling Inhibits Growth and Survival of Prostate Cancer Cells.** Fig. 5A shows that treatment of DU145 cells with LF+/Stat3 antisense oligonucleotide induced significant growth inhibition that correlated well with the inactivation of Stat3 as measured by EMSA. The control mismatch oligonucleotide had an intermediate effect on cell growth, consistent with the partial effect on Stat3 DNA-binding activity (compare Figs. 4A and 5A). This intermediate effect of the mismatch oligonucleotide may reflect nonspecific toxicity. To determine whether down-modulation of Stat3 by using antisense oligonucleotide could induce apoptosis in prostate tumor cells, we transfected DU145 cells and, 12 h later, measured apoptosis using an early marker for apoptosis, caspase 3 activation (Fig. 5B). Results demonstrate a 3-fold increase in apoptosis of the cells treated with Stat3 antisense oligonucleotide compared with the Lipofectamine-Plus (LF+) reagent alone or Stat3 mismatch control oligonucleotide. Collectively, these findings indicate that activated Stat3 signaling is required for the growth and survival of prostate cancer cells.

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**5 L. B. Mora, unpublished observations.**
cells in vivo (42), further supporting a role for activated Stat3 in prostate cancer. To our knowledge, this is the first study in which the levels of activated Stat3 have been evaluated in parallel by a biochemical assay for Stat3 DNA-binding activity and an immunohistochemical assay for phospho-Stat3 in human tumors. Furthermore, levels of Stat3 activation were evaluated in the context of clinicopathological characteristics in a group of 45 patients with primary prostate adenocarcinoma. Our analysis demonstrates that higher levels of Stat3 activation are associated with higher Gleason scores (43), which is indicative of more aggressive and poorly differentiated tumors. This observation may be valuable for identification and management of high-risk prostate cancer patients, especially those with high Gleason scores.

Earlier studies showed that activated Stat3 signaling contributes to the growth and survival of diverse human cancer cells (24) such as multiple myeloma (15), breast carcinoma (43), melanoma (44), and head and neck squamous carcinoma (45). Thus, the development of therapeutic agents that target Stat3 signaling represents a potentially new treatment approach for prostate cancer and other human tumors (46). With the determination of phospho-Stat3 levels by immunohistochemistry, it might be possible to identify and stratify patients who may respond to therapies specifically targeting Stat3 signaling. This immunohistochemical assay has certain advantages over EMSA including cost effectiveness, reproducibility, and adaptability to the clinical setting. Importantly, the specificity of this immunohistochemical assay was confirmed by specifically ablating Stat3 expression with the use of antisense Stat3 oligonucleotides. Another major advantage is that the specific cell types (i.e., tumor and nontumor cells) that have activated Stat3 can be distinguished by immunohistochemical but not by molecular approaches such as EMSA or Western blot analysis. Moreover, inexpensive and rapid follow-up of clinical outcome and response to treatment can be performed based on repeated

Fig. 4. Antisense Stat3 oligonucleotides block Stat3 protein expression, DNA-binding activity and immunohistochemical reactivity. A, Stat3 DNA-binding activity as measured by EMSA in nuclear extracts prepared from DU145 cells treated as follows: Lane 1, untreated control; Lane 2, Lipofectamine-Plus (LF+)/Stat3 antisense oligonucleotide (250 nM); Lane 4, LF+/Stat3 mismatch oligonucleotide (250 nM); and Lane 5, untreated cells with anti-Stat3 antibody (Supershift). B, Western blot analysis of total Stat3 protein expression after treatment with: Lane 1, Lipofectamine-Plus (LF+) alone; Lane 2, LF+/Stat3 mismatch oligonucleotide; and Lane 3, LF+/Stat3 antisense oligonucleotide. C, immunohistochemical staining of phospho-Stat3 (600) using cytospins from DU145 cells treated with Lipofectamine-Plus (LF+) alone, LF+/Stat3 antisense oligonucleotide, or LF+/Stat3 mismatch oligonucleotide. Different intensities of antibody immunoreactivity (specific nuclear localization) correlated with the Stat3 DNA-binding activities shown in A. The cells transfected with Stat3 antisense oligonucleotides had a completely negative nuclear immunoreactivity, demonstrating the specificity of the phospho-Stat3 immunohistochemical assay. D, immunohistochemical demonstration of induction of Stat3 activation in LNCaP cells after treatment with IL-6 (10 ng/ml at 40 min). x400.
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immunohistochemical assays using limited amounts of tissue material including core biopsies. It is important to note, however, that rapid processing (less than 15 min from surgical removal) is essential for preserving the in vivo phosphorylation state of Stat3 protein and, thus, for obtaining reliable data on Stat3 activation in tumor specimens. The immunohistochemical protocol is also critical to expose the epitope without denaturing it too much so that it can be recognized by the antibodies. In conclusion, these results encourage further exploration of the potential prognostic and therapeutic value of elevated Stat3 activation in patients with prostate carcinoma and other tumors.

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Fig. 5. Transfection with antisense Stat3 oligonucleotides induces growth inhibition and apoptosis of DU145 cells. A, DU145 prostate cancer cells were transfected with Stat3 antisense or mismatch oligonucleotides (250 nm) for 48 h and viable cell numbers were estimated by counting with a hemocytometer using trypan blue dye exclusion. B, apoptosis was assayed by flow cytometry using antibodies to activated caspase-3 after 12-h transfection with Stat3 antisense oligonucleotides. All values are expressed as means ± SDs (n = 3).

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Stat3 ACTIVATION IN PROSTATE CANCER


Constitutive Activation of Stat3 in Human Prostate Tumors and Cell Lines: Direct Inhibition of Stat3 Signaling Induces Apoptosis of Prostate Cancer Cells

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