Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines

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Abstract
Signal transducers and activators of transcription (STAT) were originally discovered as components of cytokine signal transduction pathways. Persistent activation of one STAT, STAT3, is a common feature of prostate cancer. Activated STAT3 was found in pathology specimens obtained from prostatectomy in the cancerous areas but not in the normal margins. Because the activation of STAT3 is mediated by the action of an upstream Janus kinase (JAK) kinase, usually JAK1 or JAK2, the activation step for STAT3 might itself be a target for therapy in prostate cancer. However, the redundancy of upstream kinases may make this strategy unreliable for therapy. To develop molecular targets for prostate cancer treatment, JAK kinase and STAT3 inhibition of two prostate cancer lines were compared. DU145 and NRP-154 cells were treated with JAK kinase inhibitors, analyzed for onset of apoptosis, and measured by annexin V binding and propidium iodide uptake. Activation of caspases in the cells was determined by measuring cleaved caspase-3 following treatment. For determining the effect on mitochondrial membrane depolarization that accompanies apoptosis, the fluorescent dye JC-1 was used. STAT3 was specifically inhibited by transfecting either a dominant-negative (DN) STAT3 plasmid or antisense STAT3 oligonucleotides into the cells. To look for reduction in STAT3 levels within cells, fixed and permeabilized prostate cancer cells were stained with antibody to STAT3. We found that more than one JAK kinase is involved in STAT3 activation in prostate cancer lines. AG490 (JAK2 specific) induced apoptosis in DU145 but not in NRP-154 prostate cancer lines, whereas piceatannol (JAK1 specific) induced apoptosis in NRP-154 but not in DU145 cells. Next, we demonstrated efficacy of specific STAT3 inhibitors in prostate cancer lines. Both induction of apoptosis and reduction in intracellular STAT3 protein were observed following treatment with antisense STAT3 oligonucleotides, while transfection of a DN-STAT3 plasmid into both prostate cancer cell lines resulted in loss of viability and onset of apoptosis. We conclude that STAT3-specific inhibitors, rather than JAK kinase-specific inhibitors, should be more useful therapeutically in treating androgen-resistant prostate cancer and that STAT3 is an appropriate target in the treatment of prostate cancer.

Introduction
Prostate cancer is the most frequently diagnosed non-cutaneous malignancy of males in the United States, ultimately affecting 35% of all American men (1, 2). Androgens play a critical role in the tumorigenic process, with activity mediated by the androgen receptor. Initially, prostate cancer is androgen sensitive, and most patients respond to androgen ablation therapy. However, there are side effects to this therapy that make it unpleasant for the patient (3). Even with androgen ablation therapy, the disease often recurs in androgen refractory form (4). Androgen ablation therapy may benefit the patient in the short term but apparently has no effect on relapse-free, long-term survival (3). When prostate cancer recurs, treatment consists of cytotoxic chemotherapeutic agents, which due to their high nonspecific cytotoxicity has narrow therapeutic indices and many undesirable side effects. Moreover, androgen-resistant prostate cancer does not respond well to cytotoxic agents and has a high mortality rate. Therefore, finding a therapy that (a) does not have the side effect of androgen ablation therapy and (b) is effective on the recurring, androgen-resistant tumor would have major impact on the prostate cancer patient population.

Signal transducers and activators of transcription (STAT) are proteins that regulate gene expression by affecting transcription. They are part of the signal transduction pathway of many growth factors and cytokines and are activated by phosphorylation of tyrosine and serine residues by upstream kinases (5). For example, signaling by interleukin (IL)-6 generally induces phosphorylation of STAT3 (5). In benign cells, the signaling by STAT3 is under tight regulation so that the signal is transient. However, aberrant signaling by STAT3 was reported in many types of malignancies, such as myeloma, head and neck cancer, breast cancer, and prostate cancer (6–9). Malignant cells expressing persistently activated STAT3 become dependent on it for survival; disruption of activation or expression of STAT3 resulted in apoptosis (7–11). Recently, investigators

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demonstrated that inhibition of tumor cell-expressed STAT3 inhibited vascular endothelial growth factor production by the tumor cells (12). Because vascular endothelial growth factor is one of the most potent angiogenic factors, its inhibition prevented the development of tumor neovascularization. STAT3-responsive elements were identified in several genes that influence cell cycle progression or inhibit apoptosis. These genes include cyclin D1 and Bcl-X<sub>L</sub>. The up-regulation of the expression of these genes in cancer has been well documented (13–18).

Like many forms of cancer, prostate cancer cells produce and respond to cytokine growth factors such as IL-6. Previously, investigators noted that IL-6 was involved in prostate cancer cell line survival and proliferation. In a study of 73 men with metastatic prostate cancer, high IL-6 levels in serum were observed to correlate with objective measures of morbidity (decreased hematocrit, hemoglobin, and serum cholesterol; 19). The human prostate cancer cell lines DU145 and PC3 were found to synthesize IL-6. Moreover, these lines, which are resistant to chemotherapeutic drugs such as cis-diamminedichloroplatinum and etoposide, died in the presence of a combination of those drugs and antibody to human IL-6 (20). In other words, inhibition of autocrine IL-6 signaling resulted in the restoration of the cytotoxic efficacy in DU145 and PC3 prostate cancer cell lines. Further experiments demonstrated that IL-6 is very important in the progression from androgen-sensitive prostate cancer to androgen-resistant prostate cancer. It was observed that benign prostatic hyperplasia cells exhibited little or no proliferative response to exogenous IL-6, whereas the androgen-sensitive human prostate cancer cell line LNCaP grew more rapidly when exposed to exogenous IL-6 (21) or when transfected with a constitutively activated form of the STAT3 gene, S3c (22). Antibody to IL-6 inhibited the growth of some prostate cancer cell lines (23), which were sensitive to the cytotoxic activity of an exotoxin: anti-IL-6 fusion protein (24). Finally, three human prostate cancer cell lines (PC3, DU145, and TSU) were shown to be sensitive to AG490 (31). Based on these data, we decided to investigate inhibition of STAT3 by both inhibition of activation and direct inhibition of expression to determine the best strategy of STAT3 inhibition to induce apoptosis in two prostate cancer lines.

**Materials and Methods**

**Immunohistochemistry for Phospho-STAT3 in Prostate Cancer Specimens**

Fifteen archived specimens of prostates obtained by radical prostatectomy or transurethral resection at the Division of Urology, University of Medicine and Dentistry of New Jersey University Hospital were used. These were fixed in 10% neutral-buffered formalin and embedded in 4-mm paraffin blocks; then, 4-μm serial sections were cut from them with a microtome. Initially, sections were stained with H&E for pathological diagnosis and Gleason grading, which was performed on four to six fields of areas of similar pathological conditions. The general methods and grading used were previously reported (32). Adjacent sections were used for immunohistochemistry of phosphorylated STAT3. These sections were laid on glass microscope slides followed by deparaffinization in xylene and rehydration in graded ethanol; the antigens were then retrieved by boiling the sections in 0.01 M sodium citrate (pH 6.0) for 30 min. Sections were next incubated with phosphospecific antibody to STAT3 (biotinylated rabbit antiphospho- Tyr<sup>705</sup>-STAT3; Cell Signaling, Beverly, MA) overnight at 1:1000 final dilution. Immunostaining of STAT3 was performed using the Catalyzed Signal Amplification System by procedures outlined by the manufacturer (DAKO Corp., Carpinteria, CA). For a negative control, the primary antibodies were replaced with the same antibodies that had been first incubated with 50-fold excess of purified STAT3 peptide at 4°C overnight. For each specimen, the immunostaining was performed at least twice at different times.

**Oligonucleotides**

Oligonucleotides were either obtained from Isis Pharmaceuticals or synthesized using phosphorothioate chemistry by the Molecular Resources Facility, New Jersey Medical School, University of Medicine and Dentistry of New Jersey. The sugar moieties of five bases at both 5′ and 3′ ends were modified with 2′-O-methoxymethyl groups to increase stability of the oligonucleotides and to provide higher hybridization affinity (33). For determinations of transfection efficiencies, fluorescent oligonucleotides labeled with either FITC or cyanine-3 were synthesized. Cyanine-3-modified oligonucleotides were made by the Molecular Resources Facility while the FITC-labeled oligonucleotides were from Isis Pharmaceuticals. The antisense and sense sequences
used in the experiments were 5'-GCT CCA GCA TCT GCT GCT TC-3' and 5'-GAA GCA GCA GAT GGA GC-3', respectively (30). The sequence of the fluorescent control oligonucleotide was 5'-GTG CTC ATG GTG CAC GGT CT-3'.

**DN-STAT3 and cSTAT3 Genes**

The STAT3β gene has been described to function as a DN form of the STAT3 gene, inhibiting expression of STAT3 (6, 8, 11, 34). The STAT3β gene on pIRES-EGFP (DN-STAT3; gift of Dr. Richard Jove, University of South Florida) was transfected using Clonfectin (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer’s protocol. The vector pIRES-EGFP (Clontech Laboratories) was used as a control.

**Cells**

NRP-154 cells were the gift of Dr. David Danielpour, Case Western Reserve School of Medicine. DU145 cells were the gift of Dr. Linda Mora, University of South Florida. They were maintained in either DMEM:Ham’s F-12 or RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% newborn bovine serum (Hyclone Laboratories, Logan, UT). Cell viability was determined using fluorescein diacetate (FDA; 35) and a Zeiss (Jena, Germany) Universal RII fluorescence microscope.

**Transfection of Oligonucleotides into Prostate Cancer Lines**

LipofectAMINE2000 transfection reagent (Invitrogen) was used to transfect oligonucleotides into the prostate cancer lines. We routinely observed greater than 85% transfection efficiency using this protocol (Fig. 2). Briefly, cells plated in six-well plates were grown to 50–70% confluency. Oligonucleotides were diluted in OptiMEM I (Invitrogen) appropriately. Next, LipofectAMINE2000 was diluted in OptiMEM I at 2 μl LipofectAMINE2000 to 250 μl OptiMEM I/well. The diluted LipofectAMINE2000 was allowed to incubate for 5 min at room temperature and then added to cells in 250 μl of diluted oligonucleotide. The liposome-oligonucleotide mixture remained at room temperature for 20 min before the 500 μl of liposome-oligonucleotide mixture was added to each well of cells. Cells were incubated with the liposome-oligonucleotide mixture for 6 h at 37°C and 1.5 ml/well of medium containing 30% serum were added to each well. Additional medium containing 10% serum was added each of the following days until the experiment was ended.

**Treatment of Prostate Cancer Lines with JAK Inhibitors**

Piceatannol and AG490 were obtained from Calbiochem (San Diego, CA) and were dissolved in cell culture medium and used at 100 μM final concentration. Subconfluent cultures of DU145 and NRP-154 cells in six-well plates were treated with the compounds for 48 and 72 h; then, cells were harvested and stained with FITC-Annexin V and propidium iodide (PI) to look for apoptosis by flow cytometry (see below).

**Transfection of DN-STAT3 into Prostate Cancer Lines**

Clonfectin was used to transfect the DN-STAT3 plasmid and the empty vector pIRES-EGFP into the prostate cancer lines. Clonfectin, heated to 65°C, was diluted 1:100 in OptiMEM I medium and then added to 90 μl containing 1–2 μg of either vector or DN-STAT3. After vigorous vortexing, the liposomes were allowed to form for 1 h at room temperature and then added to cells in 250 μl of OptiMEM I medium (in six-well plates). Four hours later, the cell-free liquid was aspirated and replaced with the usual cell growth medium.

**Apoptosis Determinations**

FITC or phycoerythrin (PE) annexin V and PI staining [Caltag (Burlingame, CA) and PharMingen (La Jolla, CA)] were used to measure the induction of apoptosis by the test compounds after incubation for the appropriate amount of time. Harvested cells were washed twice in buffer; then, 5 × 105 cells in 1 ml of buffer, containing at least 40 mM Ca2+, were put into each tube (Falcon, Franklin Lakes, NJ). Five microliters of fluorescent-annexin V and of PI were put into each tube. Fluorescence was quantified on a Becton Dickinson (San Jose, CA) FACScan for at least 10,000 events.

The JC-1 dye assay was used for determination of reduction in mitochondrial membrane potential during apoptosis (36, 37). Briefly, 106 cells in 1 ml were stained with 1 μl of JC-1 (Molecular Probes, Eugene, OR) at 1 mg/ml in DMSO according to manufacturer’s instructions at various times following treatment with sense or antisense STAT3 oligonucleotides or DN-STAT3. After incubation with JC-1 for 15 min at 37°C, cells were analyzed for the decrease in red-orange fluorescence on a FACScan flow cytometer; at least 10,000 events were collected for analysis.

To measure caspase activation, the caspase-3 kit from Becton Dickinson PharMingen (La Jolla, CA) was used according to the manufacturer’s directions. The PE-labeled antibody in this kit recognizes the active (cleaved) form of caspase-3, and a labeled control immunoglobulin was used to determine the amount of nonspecific staining in each experiment. Treated cells were harvested at various times after transfection, fixed, and permeabilized with the reagents in the kit. Cells were incubated with PE-labeled antiactivated caspase-3 antibody or with labeled control immunoglobulin for 1 h on ice and then washed thrice with wash buffer from the kit. Fluorescence was analyzed on a FACScan flow cytometer for at least 10,000 events.

To confirm that caspase-3 was activated in the apoptotic pathway by treatment with antisense STAT3 oligonucleotide, the irreversible caspase-3 inhibitor Z-DEVD-FMK (Ready-to-Use form; Alexis Biochemicals, San Diego, CA) was added at 100 μM for 10 min prior to the addition of antisense oligonucleotide (38, 39). The cells were incubated with antisense and control oligonucleotides for 48 h and then were harvested and stained with FITC-Annexin V and PI prior to fluorescence analysis on the FACScan.

**Determination of Reduction in Intracellular STAT3**

We used intracellular staining and flow cytometry to determine the amount of activated STAT3 present in cells
(25, 40) and confirmed the decrease in STAT3 protein by Western blot. For intracellular flow cytometry, briefly, 24 h after treatment with antisense oligonucleotides, cells were fixed in 4% paraformaldehyde/PBS for 20 min, permeabilized with 0.1% saponin/PBS for 1 h, and incubated with goat immunoglobulin for 1 h. Cells were washed with 0.1% saponin/PBS throughout the remainder of the procedure. Rabbit anti-STAT3 antibody or control rabbit immunoglobulin (Sigma Chemical Co., St. Louis, MO) was preincubated with PE-labeled anti-rabbit IgG (Zenon R-PE rabbit labeling kit; Molecular Probes) and then incubated with cells for 1 h. After extensive washing, the fluorescence of at least 10,000 events was quantified by the FACSscan flow cytometer.

To confirm our findings of reduced STAT3 levels in cells treated with antisense STAT3 oligonucleotides, we used Western blot hybridization. Replicate cultures of treated prostate cancer cells were lysed with commercial lysis buffer (Pierce Biotechnology, Rockford, IL). The protein species were separated by gel electrophoresis using 10% Novex (Invitrogen) precast gels, buffer, and apparatus followed by Western blot hybridization on nylon Millipore (Billerica, MA) membranes. The membranes were blocked with 2% BSA prior to incubation with rabbit antibody to STAT3 followed by incubation with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin. Bands were visualized using the chemiluminescence system of Amersham (Piscataway, NJ) and Kodak (Rochester, NY) film.

Statistical Analysis

The software program Kaleidograph (Synergy Software, Essex Junction, VT) and the graphing program InStat3 (GraphPad Software, San Diego, CA) were used for data analyses.

Results

Activated STAT3 Was Found in Prostate Cancer Specimens but not in Normal Tissue

To determine whether there is activated (phosphorylated) STAT3 present in prostate cancer in vivo, we obtained archived specimens from resected prostates of 15 patients. The sections had previously been graded for severity of prostate cancer by a pathologist at New Jersey Medical School (32). Fifteen out of 15 archived specimens (100%) stained with antibody to phospho-STAT3; moreover, the staining was most intense in the areas of cancerous tissue and was much less intense or not present in the normal tissue margins. Fig. 1 shows the results obtained for 2 of the 15 specimens stained in this way for phospho-STAT3. Fig. 1A shows an area of high-grade cancer, with concomitant dense staining for phospho-STAT3 (arrows). Fig. 1B shows a specimen from a different patient with low-grade prostate cancer. The normal margin shows no to very little staining for phospho-STAT3, while the area of the low-grade cancer shows moderate staining for phospho-STAT3 (arrows). Therefore, activated STAT3 is present in vivo in prostate cancer and correlated with the severity of the malignancy within the resected tumor.

Differential Effects of JAK Inhibitors on Prostate Cancer Cell Lines

Our initial studies used AG490, described as a STAT3 inhibitor (28, 41, 42), induced apoptosis in human DU145 but not in rat NRP-154 prostate cancer cells (25, 31). We extended our studies in rat NRP-154 prostate cancer cells to include another inhibitor, piceatannol (43). The results are shown in Table 1. We found that NRP-154 cells were resistant to AG490 at 100 μM but sensitive to piceatannol at the same concentration, whereas DU145 cells were sensitive to AG490, as reported, and resistant
to piceatannol. These two concentrations have very narrow ranges of efficacy; we had not previously observed effects at 30 or 50 μM for either compound, nor have other investigators (28, 31, 43, 44).

AG490 is not a STAT3 inhibitor per se; it is an inhibitor of JAK2 kinase activity and consequently inhibits the activation of STAT3. Piceatannol is a JAK1 kinase inhibitor. We concluded from these studies that NRP-154 cells signal via JAK1 to activate STAT3, a distinct difference from the signaling pathway reported in human prostate cancer lines such DU145, and that the upstream kinases that activate STAT3 may not be the best molecular targets in prostate cancer. Therefore, methods of delivering STAT3-specific inhibitors to prostate cancer cells were investigated: inhibition of STAT3 gene expression by antisense oligonucleotides to STAT3 and by DN-STAT3 plasmid.

Antisense STAT3 Oligonucleotides Induced Apoptosis in Prostate Cancer Cells

We used a cyanine-3-labeled control sequence to examine oligonucleotide uptake by our prostate cancer cell lines. We determined that over 90% of the DU145 cells fluoresced when transfected with 125 nM final concentration of labeled oligonucleotide in most experiments, and similar results were obtained with NRP-154 cells (data not shown). At least 85% of DU145 and NRP-154 cells

Table 1. Effect of JAK1 and JAK2 inhibitors on prostate cancer cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Drug</th>
<th>μM</th>
<th>% Apoptotic ± SD</th>
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<tbody>
<tr>
<td>NRP-154</td>
<td>AG490</td>
<td>0</td>
<td>8 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>7.5 ± 5</td>
</tr>
<tr>
<td></td>
<td>Piceatannol</td>
<td>100</td>
<td>56 ± 12*</td>
</tr>
<tr>
<td>DU145</td>
<td>AG490</td>
<td>100</td>
<td>0.5 ± 3</td>
</tr>
<tr>
<td></td>
<td>Piceatannol</td>
<td>100</td>
<td>64 ± 12*</td>
</tr>
</tbody>
</table>

*P < 0.001, two-tailed t test.

Notes: Cells (30–50% confluent in six-well plates) were treated with AG490, piceatannol, or vehicle as described in Materials and Methods. Forty-eight hours after treatment, cells were harvested and apoptosis was measured using FITC-annexin V and PI on a FACScan flow cytometer; 10,000 events were collected from each sample for analysis.

Figure 2. Treatment of DU145 prostate cancer cells with antisense STAT3 oligonucleotide induced apoptosis. DU145 cells were transfected with oligonucleotides using LipofectAMINE2000 according to the manufacturer’s instructions (see Materials and Methods). A, photomicrograph showing perinuclear localization of fluorescently labeled antisense oligonucleotide 24 h after transfection. Magnification is ×100. B and C, density plots of 10,000 events showing apoptosis induced by STAT3 antisense oligonucleotide in DU145 cells. Cells were stained with FITC-annexin V and PI and then analyzed at 48 h after transfection on a FACScan flow cytometer. B, 62.5 nM STAT3 antisense. C, 125 nM STAT3 antisense. Treatment with 0 nM antisense (mock transfection; lipid reagent only) did not result in significant apoptosis (≤5%; data not shown). D and E, DU145 cells transfected with (D) 200 or (E) 500 nM sense STAT3 oligonucleotide at 48 h and stained with FITC-annexin V and PI.
were transfected using LipofectAMINE2000 in the experiments carried out thus far. In every experiment performed, the transfection efficiencies were determined to insure that cells were actually transfected in each experiment.

Next, the effect of a STAT3 antisense oligonucleotide on apoptosis in DU145 cells was examined. It was observed that significant apoptosis was induced by the STAT3 antisense oligonucleotide, which was measurable at 48 h following transfection (Fig. 2). To demonstrate that the induction of apoptosis was sequence specific, a sense STAT3 oligonucleotide as a control (10) was used in the same experiments. Fig. 2 shows that DU145 cells were susceptible to treatment with antisense STAT3 while transfection of the sense oligonucleotide had no significant effect on DU145 viability. Fig. 2, B and C, show the results of transfecting 62.5 nM (Fig. 2B) or 124 nM (Fig. 2C) into DU145 cells at 48 h. Treatment with 62.5 nM antisense STAT3 oligonucleotide induced apoptosis in ~38% of the cells (Fig. 2B), while 125 nM induced apoptosis in over 80% of the cells (Fig. 2C). Fig. 2, D and E, show that transfection with the same concentrations of sense oligonucleotides did not induce significant apoptosis (Fig. 2D, 62.5 nM sense STAT3 oligonucleotide, shows 12% apoptosis; Fig. 2E, 125 nM sense STAT3 oligonucleotide, shows 15% apoptosis in DU145 cells). Similar results were obtained in NRP-154 cells (data not shown).

To demonstrate that prostate cancer cell lines underwent apoptosis associated with decreased mitochondrial membrane potential when transfected with antisense to STAT3, the fluorescent dye JC-1, which measures mitochondrial membrane potential, was used (37). The results are presented in Table 2. Transfection with 200 nM antisense, but not sense, oligonucleotide for 48 h decreased JC-1 fluorescence by ~25% in NRP-154 cells and by ~37% in DU145 cells. At 72 h following transfection with 200 nM STAT3 antisense oligonucleotide, the decreases in JC-1 fluorescence were even more marked: JC-1 fluorescence was decreased by over 33% in NRP-154 cells and by 50% in DU145 cells. These data reveal that antisense, but not sense, to STAT3 was capable of decreasing mitochondrial membrane potential in prostate cancer cells, which is something that occurs in apoptotic cells in many systems. It must be noted that NRP-154 cells were less sensitive than DU145 cells to antisense STAT3 oligonucleotide because the NRP-154 cells are rat cells and the antisense STAT3 oligonucleotide sequence was optimized for human STAT3.

To determine if activation of caspases was involved in the apoptotic pathway induced by treatment with antisense STAT3 oligonucleotides, the caspase-3 kit from Becton Dickinson PharMingen was used. The antibody included in the kit recognizes the active form of human caspase-3 but not the precursor form. DU145 cells transfected with 200 nM antisense or sense STAT3 oligonucleotides were harvested, fixed, and permeabilized then stained with antibody to activated caspase-3 48 h after transfection. The results are shown in Fig. 3. In both panels, the thick line shows the mean fluorescence intensity of DU145 cells stained with PE-labeled antiactivated caspase-3, while the thin line shows the mean fluorescence intensity of cells stained with the labeled control immunoglobulin. When transfected with 200 nM antisense STAT3 oligonucleotide, DU145 cells exhibited activated caspase-3 in nearly 88% of the cells (Fig. 3A); in contrast, when transfected with 200 nM sense STAT3 oligonucleotide, fewer than 10% of the DU145 cells exhibited activated caspase-3 (Fig. 3B). In Fig. 3, C and D, we present the results of DU145 cells transfected with antisense STAT3 oligonucleotides after 1 h pretreatment with 100 μM Z-DEVD-FMK, an irreversible caspase inhibitor. Without Z-DEVD-FMK treatment, we observed 61% apoptotic cells 48 h after antisense transfection (Fig. 3C), however, with Z-DEVD-FMK pretreatment, the amount of apoptosis was reduced to 22% (Fig. 3D). These results demonstrate that treatment with antisense STAT3 oligonucleotide induced a caspase-dependent apoptosis pathway in DU145 prostate cancer cells.

We concluded from these results that STAT3 is a more appropriate target for androgen-resistant prostate cancer than upstream JAK kinase inhibition and is worthy of further evaluation.

### Antisense STAT3 Reduced Intracellular Levels of STAT3 Protein in Prostate Cancer Cell Lines

Because we observed that gel shift assays were not sensitive enough to detect STAT3 activity in transfected cells overexpressing activated STAT3, we use intracellular staining and flow cytometry instead to determine the amount of activated STAT3 present in cells. We and others have found that intracellular staining/flow cytometry is both more sensitive and more quantifiable than Western blotting (25, 40). To demonstrate the effect of antisense oligonucleotides on intracellular STAT3 protein, intracellular flow cytometry to quantify the amount of STAT3 protein was employed using PE-labeled antibodies to STAT3 (25).

### Table 2. Decreased mitochondrial membrane potential during apoptosis induced by antisense STAT3 in prostate cancer lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>nM</th>
<th>Oligo</th>
<th>%JC-1+ at 48 h</th>
<th>%JC-1+ at 72 h</th>
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<tr>
<td>NRP-154</td>
<td>0</td>
<td>None</td>
<td>97.6</td>
<td>87.7</td>
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<tr>
<td></td>
<td>200</td>
<td>Antisense</td>
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<td></td>
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<td>Sense</td>
<td>68.8</td>
<td>68.5</td>
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<tr>
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<td>200</td>
<td>Sense</td>
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<td>81.1</td>
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<td></td>
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<td>Sense</td>
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<td>84.5</td>
</tr>
<tr>
<td>DU145</td>
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<td>None</td>
<td>97.3</td>
<td>92.7</td>
</tr>
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<td></td>
<td>200</td>
<td>Antisense</td>
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<td></td>
<td>500</td>
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Notes: Cells (30–50 confluent in six-well plates) were transfected with oligonucleotide using LipofectAMINE2000 as described in Materials and Methods. Forty-eight and 72 h after transfection, cells were harvested and incubated with JC-1 as described. Fluorescence intensities were determined using a FACScan flow cytometer. Percent JC-1-positive cells were the cells displaying red-orange fluorescence in the FL2 detector at channel 100 and beyond. Data shown are from a representative experiment; in each experiment, 20,000 events were collected for further analysis. NRP-154 cells are rat prostate cancer cells; therefore, they were less sensitive to the antisense STAT3 oligonucleotide that was based on the human sequence.
This method has been used by other investigators to quantify intracellular protein better than Western blot technique (40). Fig. 4 shows a histogram describing the effect of treating DU145 cells with various amount of antisense STAT3 oligonucleotides for 24 h. The thin solid black line shows the mean fluorescence intensity of cells transfected with 200 nm antisense STAT3 oligonucleotide. Thin gray line, cells stained with labeled control immunoglobulin; thick gray line, cells stained with labeled anticleaved caspase-3; M1, region of positive fluorescence. In this experiment, 87.7% of the cells were positive for activated caspase-3. B, DU145 cells transfected with 200 nm sense STAT3 oligonucleotide; 9.8% of the cells stained for activated caspase-3. Thin gray line, cells stained with labeled control immunoglobulin; thick gray line, cells stained with labeled anticleaved caspase-3. C and D, caspase inhibitor Z-DEVD-FMK pretreatment of DU145 cells inhibited antisense-induced apoptosis. DU145 cells were pretreated for 10 min with 100 μM Ready-to-Use Z-DEVD-FMK prior to transfection with 250 nm antisense STAT3 oligonucleotide. Forty-eight hours later, cells were analyzed for apoptosis using FITC-Annexin V and PI. C, cells transfected but not treated with Z-DEVD-FMK; 56% of cells stained with FITC-annexin V and/or PI. D, same as C, but cells were pretreated for 1 h with Z-DEVD-FMK. Only 22% of the cells were apoptotic when pretreated with the caspase inhibitor Z-DEVD-FMK.

ADN-STAT3 Plasmid Induced Apoptosis in Prostate Cancer Cell Lines

To confirm our results with antisense STAT3, we used a DN-STAT3 plasmid to transfect DU145 and NRP-154 (7). Previously, this gene was used to study the effect of reduction of STAT3 gene expression in various cell types. Here, it was used to see if apoptosis resulted in prostate cancer cell lines when STAT3 expression was disrupted. As reported in Table 3, transfection of DN-STAT3 induced apoptosis in NRP-154 and DU145 cells whereas transfection of the vector pIRES-EGFP did not. At 48 h after transfection,
antisense STAT3 oligonucleotide. 

To study the effects of antisense STAT3 oligonucleotides, a reproducible method for high efficiency transfection was employed using LipofectAMINE2000 in DU145 and NRP-154 cells. Efficiencies of at least 85%, as assessed using fluorescent oligonucleotides (Fig. 2), were obtained for every experiment presented. Transfection of antisense STAT3 into DU145 human prostate cancer cells induced apoptosis as measured by annexin V binding and uptake of PI (Fig. 2). Transfection of as little as 62.5 nM antisense STAT3 oligonucleotide induced apoptosis at 48 h (Fig. 2B), whereas transfection of up to 500 nM sense STAT3 oligonucleotide did not (Fig. 2, D and E). Induction of apoptosis by STAT3 antisense oligonucleotides was accompanied by decreased mitochondrial membrane potential as measured by JC-1 fluorescence (Table 2). Apoptosis in DU145 cells also correlated with activation of caspase-3 (Fig. 3); no activation of caspase-3 was observed in cells treated with sense oligonucleotides. Intracellular protein levels of STAT3 were decreased by treatment with antisense STAT3 oligonucleotide, intracellular STAT3 protein levels were reduced by over 60% in DU145 cells whereas treatment with sense oligonucleotide did not (Fig. 2, D and E). Induction of apoptosis by STAT3 antisense oligonucleotides was accompanied by decreased mitochondrial membrane potential as measured by JC-1 fluorescence (Table 2). Apoptosis in DU145 cells also correlated with activation of caspase-3 (Fig. 3); no activation of caspase-3 was observed in cells treated with sense oligonucleotides. Intracellular protein levels of STAT3 were decreased by treatment with antisense STAT3 oligonucleotide, intracellular STAT3 protein levels were reduced by over 60% in DU145 cells whereas treatment with the sense oligonucleotide did not affect the amount of STAT3 observed (Fig. 4). Furthermore, transfection of a DN form of STAT3, DN-STAT3, into NRP-154 and DU145 cells

Discussion

We have shown that STAT3 is present in cancerous areas of prostate but not in the normal margins, that STAT3 is apparently essential for prostate cancer cell survival, and that inhibition of STAT3 may be appropriate for the treatment of prostate cancer.

Because either JAK1 or JAK2 may activate STAT3 in prostate cancer cell lines (Table 1), inhibitors directed against these kinases would not be useful for treating prostate cancer because knowledge about which enzyme to inhibit would not be readily available to the physician. Therefore, the downstream target of JAK1 and JAK2, STAT3, was examined to determine if it is a molecular target in prostate cancer lines.

To study the effects of antisense STAT3 oligonucleotides, a reproducible method for high efficiency transfection was employed using LipofectAMINE2000 in DU145 and NRP-154 cells. Efficiencies of at least 85%, as assessed using fluorescent oligonucleotides (Fig. 2), were obtained for every experiment presented. Transfection of antisense STAT3 into DU145 human prostate cancer cells induced apoptosis as measured by annexin V binding and uptake of PI (Fig. 2). Transfection of as little as 62.5 nM antisense STAT3 oligonucleotide induced apoptosis at 48 h (Fig. 2B), whereas transfection of up to 500 nM sense STAT3 oligonucleotide did not (Fig. 2, D and E). Induction of apoptosis by STAT3 antisense oligonucleotides was accompanied by decreased mitochondrial membrane potential as measured by JC-1 fluorescence (Table 2). Apoptosis in DU145 cells also correlated with activation of caspase-3 (Fig. 3); no activation of caspase-3 was observed in cells treated with sense oligonucleotides. Intracellular protein levels of STAT3 were decreased by treatment with antisense STAT3 oligonucleotide, intracellular STAT3 protein levels were reduced by over 60% in DU145 cells whereas treatment with the sense oligonucleotide did not affect the amount of STAT3 observed (Fig. 4). Furthermore, transfection of a DN form of STAT3, DN-STAT3, into NRP-154 and DU145 cells

Table 3. Transfection of DN-STAT3 induced apoptosis in prostate cancer cell lines

<table>
<thead>
<tr>
<th>Cell</th>
<th>Plasmid</th>
<th>No. wells</th>
<th>No. viable cultures</th>
<th>% Apoptotic ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRP-154</td>
<td>pIRES-EGFP</td>
<td>15</td>
<td>15/15</td>
<td>7.1 ± 3</td>
</tr>
<tr>
<td>DU145</td>
<td>pIRES-EGFP</td>
<td>15</td>
<td>15/15</td>
<td>12.8 ± 4</td>
</tr>
<tr>
<td></td>
<td>DN-STAT3</td>
<td>15</td>
<td>0/15</td>
<td>75.6 ± 10*</td>
</tr>
</tbody>
</table>

Notes: NRP-154 and DU145 cells were grown in six-well plates until ~50% confluent, at which time they were transfected with DN-STAT3 or pIRES-EGFP as described in Materials and Methods. Viability of the cell cultures was assessed visually using a Zeiss Universal III fluorescence microscope and FDA to stain the cells and then counting on a hemocytometer after removing cells from the plate. The number of viable cultures refers to the number of transfected cultures that could be propagated continuously for more than 6 weeks. Apoptosis at 48 h was measured using FITC-annexin V and PI on a FACScan flow cytometer as described in Table 2 and Materials and Methods. The results of three independent experiments are shown.
induced apoptosis (Table 3), again demonstrating that STAT3 expression is necessary for survival of prostate cancer cell lines. We routinely observed less apoptosis following transfection with the DN-STAT3 plasmid than with antisense oligonucleotides; we believe the difference was due to different transfection efficiencies between the two methods used to transfect the cells.

We and others (30, 45) have demonstrated that activated STAT3 is present in prostate cancer. Whether prostate cancer cell survival requires STAT3 has been recently questioned; the data presented here indicate that STAT3 is needed for prostate cancer cell survival. In agreement with Mora et al. (30) and Ni et al. (31), we observed that apoptosis was induced when STAT3 was inhibited by either DN-STAT3 or antisense STAT3 oligonucleotide. Moreover, we extended their studies to quantify apoptosis by examining annexin V binding and PI uptake with decreased JC-1 staining and with activation of caspase-3. We showed that annexin V binding is accompanied by decreased mitochondrial membrane potential and by activation of caspase-3, all of which indicate that prostate cancer cell survival is dependent on activated STAT3. One of the protein targets of caspase-3 is Bcl-2, an antiapoptotic protein known to be under STAT3 regulation (46–48). We are in the process of using a cSTAT3-bearing plasmid in benign prostatic hyperplasia cells to find out which genes are activated by STAT3 in prostate cancer. This information should give us new insight into how STAT3 induces and maintains the tumorigenic state.

Our data show that inhibition of STAT3 by transfection of appropriate oligonucleotides is an effective and feasible approach to prostate cancer therapy. The inhibitors chosen induced apoptosis in the prostate cancer lines grown in our laboratory. Some other recent studies in prostate cancer have shown some efficacy with antisense oligonucleotides to the antiapoptotic protein Bcl-2 and to protein kinase C (49, 50). In other prostate cancer research, investigators used antisense to the antiapoptotic testosterone-repressed message 2 gene. They observed that the antisense oligonucleotide was not effective against established tumors but rather was synergistic with androgen therapy (51), making our approach to treating prostate cancer novel. We think inhibition of STAT3 may be appropriate especially for androgen-resistant prostate cancer because other investigators have noted that LNCaP cells transfected with cSTAT3 lose the response to androgens (22).

It is highly feasible to target tumors by using antisense to required signaling molecules. As the success of Gleevec shows, signaling molecules can be inhibited in cancer, with therapeutically desirable results (52). The use of antisense technology should make it possible to design easily the specific oligonucleotide inducing apoptosis in individual patient’s cancers and tumors. We hope to extend our in vitro studies in the future to examine the feasibility of in vivo delivery of STAT3 antisense oligonucleotides to localized and metastatic prostate tumors.

We are continuing our studies using molecular inhibitors of STAT3 as a way to probe the relationship between androgen receptor expression and persistently activated STAT3 (27, 53, 54). We have preliminary evidence showing a direct relationship between persistently activated STAT3 and androgen resistance in prostate cancer cell lines (Barton et al., manuscript in preparation). Completing this work will allow us to evaluate the potential usefulness of STAT3-targeted gene therapy in prostate cancer therapy more fully. In conclusion, the data presented here support STAT3 targeting for the treatment of prostate cancer.

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References


Molecular Cancer Therapeutics

Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines

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