Research article

IL-6 signaling by STAT3 participates in the change from hyperplasia to neoplasia in NRP-152 and NRP-154 rat prostatic epithelial cells

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Abstract

Background: STAT3 phosphorylation is associated with the neoplastic state in many types of cancer, including prostate cancer. We investigated the role of IL-6 signaling and phosphorylation of STAT3 in 2 rat prostatic epithelial lines. NRP-152 and NRP-154 cells were derived from the same rat prostate, yet the NRP-152 cells are not tumorigenic while the NRP-154 cells are tumorigenic. These lines are believed to represent 2 of the stages in the development of prostate cancer, hyperplasia and neoplasia. Differences in signaling pathways should play a role in the 2 phenotypes, hyperplastic and neoplastic.

Methods: We looked at the phosphorylation state of STAT3 by intracellular flow cytometry, using phospho-specific antibodies to STAT3. We used the same method to examine IL-6 production by the cell lines. We also measured apoptosis by binding of fluorescent annexin V to the cells.

Results: Although both cell lines made IL-6 constitutively, phosphorylated-STAT3 was present in untreated NRP-154 cells, but not in NRP-152 cells. Treatment with dexamethasone inhibited the IL-6 production of NRP-152 cells, but enhanced that of NRP-154 cells. Treatment with the JAK2 inhibitor AG490 induced apoptosis in NRP-152, but not NRP-154 cells.

Conclusions: We conclude from these experiments that STAT3 activity plays a role in the phenotype of NRP-154 cell, but not NRP-152 cells. The significance of alternative IL-6 signaling pathways in the different phenotypes of the 2 cell lines is discussed.

Background

Prostate cancer (PCA) is the leading cause of death in the American male over age 55, according to recent data [1]. To date, the mechanisms underlying the pathogenesis of this disease, including how normal prostate cells become neoplastic, remain unidentified. Moreover, the treatment efficacy of this disease remains limited, especially when it recurs. A thorough understanding of the neoplastic process could facilitate earlier detection of the disease, lead to more specific therapies for PCA, and ultimately improve survival.

PCA is one of several types of cancers in which IL-6 has been found or is thought to play a pathophysiological role. Some researchers think IL-6 may play a role in PCA because of what IL-6 does in other model systems of can-
As mentioned above, IL-6 is a cytokine that functions as a necessary growth factor in several cancer types, most studied in multiple myeloma [12]. It is an essential factor in the development and maintenance of B cell neoplasms [13], and likely plays an important role in many other types of cancer. IL-6 signals through a set of signaling proteins of the JAK and STAT kinase families [14]. The JAK and STAT kinases are activated by phosphorylation initiated by the homodimerization of the IL-6/IL-6 receptor complex on the cell surface. The major IL-6 signaling intermediates are JAK2 and STAT3 [15]. Homodimerization of the IL-6 receptor complex induces the autophosphorylation of JAK2. The now-activated JAK2 phosphorylates STAT3, which forms homodimers, can cross the nuclear membrane and function as a transcription factor, inducing various genes including genes involved in the cellular transformation process [15].

An association between autocrine IL-6 and PCA has been known for some time [16,17]. The change in prostate cell phenotype from paracrine IL-6-stimulated to autocrine IL-6-stimulated is believed to be a contributing factor in the progression from benign hyperplasia to neoplasia [17]. IL-6 is also implicated in the development of cancer cell resistance to chemotherapy in PCA patients [18,19]. In other studies, a chimeric protein consisting of an anti-IL-6 Ab fused to Pseudomonas exotoxin was found to inhibit proliferation of prostate carcinoma cell lines [20]. Exogenous IL-6 activated androgen responsive gene expression in the absence of androgens in human LNCaP cells [21]. More work is needed to clarify the role of IL-6 in prostate neoplasia.

While there is some evidence suggesting IL-6-mediated neoplasia in PCA development [17,22], a system suitable for following the transformation of prostate cells during PCA development remains lacking. We chose to use the NRP-152 and NRP-154 cell lines, derived by Danielpour, et al. [23], to examine the question of IL-6-mediated neoplastic progression via STAT3 activation. The 2 lines were derived from the same part of the rat prostate, following treatment in vivo with N-methyl-N-nitrosourea.

The NRP-152 cells are immortalized but not transformed, require several growth factors for in vitro survival, and do not give rise to tumors in vivo. The NRP-154 cells are transformed, grow in the absence of exogenous growth factors, and are tumorigenic [23–27]. These lines come from epithelial cells. While prostatic epithelium is resistant to neoplastic transformation, it is not resistant to the development of hyperplasia. Studying the neoplastic transformation events in a cell type inherently resistant to this type of change can yield much valuable information about the transformation process in prostate cells.

Materials & Methods

Cell lines and growth media

The tumorigenic (NRP-154) and non-tumorigenic (NRP-152) rat prostate epithelial cell lines were the gift of Dr. David Danielpour, Ireland Cancer Center, University Hospital of Cleveland, Case Western Reserve University, Cleveland, OH [23]. NRP-152 cells were propagated in DMEM/ Ham's F12 medium (1:1; GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 2 mM glutamine (GIBCO), epidermal growth factor (20 ng/ml), insulin (5 µg/ml), dexamethasone (0.1 mM) and cholera toxin (10 µg/ml); all reagents listed, Sigma), pH 7.3. NRP-154 cells were grown in DMEM/F12 plus serum and dexamethasone only. Both lines were grown in a humidified 37°C CO2 incubator until the monolayers reached about 90% confluence. For treatment with steroids, the cells were cultured in complete medium, in which the serum was replaced by charcoal-stripped serum overnight. Cells were treated with 20 nM testosterone for 6 hr. The cells were harvested with trypsin/EDTA solution, washed, and subjected to further analyses.

Intracellular flow cytometry for analysis of IL-6 and phospho-STAT3

NRP-152 and NRP-154 cells were grown as described above. For analysis of IL-6 production, the cells were fixed in Cytofix (Pharmingen) for 30 min on ice, then washed and permeabilized with Cytoperm (Pharmingen) for 15 min on ice. After washing with Perm/Wash buffer (Pharmingen), cells were incubated in 5–10 mg/ml goat Ig for 1 hr on ice. Cells were washed three times in Perm/Wash buffer, then incubated with 1 µg biotinylated anti-rat IL-6 (Pharmingen)/10⁶ cells in 100 μl Perm/Wash buffer for 1 hr on ice. After washing with Perm/Wash buffer three times (first wash being a 1 hr period in which the cells remain in Perm/Wash buffer for 1 hr on ice), cells were incubated with phycoerythrin-labeled streptavidin (Pharmingen) for 1 hr on ice, and washed three times as described for the Ab incubation step, then brought to 1 ml with PBS [28].
For analysis of phospho-STAT3, a different method was used to visualize the phosphorylated protein species. NRP-152 and NRP-154 cells were grown in the presence or absence of testosterone, as described above. Cells were fixed in Fix & Perm Medium A (Caltag) for 10 min at room temperature. After washing twice in PBS, cells were resuspended in ice-cold methanol with vortexing, then allow to sit for 15 min on ice. After washing twice in PBS, cells were resuspended in Fix & Perm Medium B (Caltag) and allowed to remain at room temperature for 30 min. Medium B contained 2 mg/ml goat Ig for blocking non-specific binding. After washing three times (including a 30 min time in cold PBS for the first wash), the cells were incubated with rabbit anti-phospho-STAT3 (Biosource), 1 µg Ab/10^6 cells in 100 µl buffer. The Ab is specific for the phosphorylated form of STAT3; it does not bind to unphosphorylated STAT3 or to other phosphorylated signaling intermediates. After incubating for 1 hr on ice, the cells were washed, with a long period in PBS for the first wash as described above. Next cells were incubated with phycoerythrin-labeled goat anti-rabbit F(ab)_2' (Caltag) for 1 hr on ice, and washed as described. For analysis, cells were brought to 1 ml in PBS. All flow cytometric analyses were performed on a Becton-Dickinson FACScan, using CellQuest software for acquisition and analysis.

**Treatment of NRP-152 and NRP-154 cells with dexamethasone**

NRP-152 and NRP-154 cells or clones (see below) were seeded at 10^5 cells/well in microtiter plates in the presence or absence of dexamethasone (Sigma) at 0.1 and 1 µM. After 48 hr, NRP-152 and NRP-154 cells replicate wells of cells were harvested with either trypsin/EDTA (GIBCO) or 0.15 M NaCl/ 0.01 M Na citrate buffer (citrate-saline buffer), and the cells were processed for intracellular flow cytometry to analyze IL-6 production, as described above.

**Cloning NRP-154 cells by limit-dilution**

Washed NRP-154 cells were diluted to 10 cells/ml in complete medium, and 100 l/well of diluted cells were placed in wells of a microtiter plate. An additional 100 µl/well complete medium were added, and the cells were incubated until growth was noted, 10 days later. At that time, 16/96 wells had cells growing in them (16.7% cloning efficiency), while the remaining wells did not. Medium was replaced, and plate was incubated until cells had grown enough to be removed to bigger wells. Clones were expanded, then analyzed for IL-6 production, as described above.

**Analysis of NRP-152 and NRP-154 cells for expression of IL-6 receptor**

Harvested NRP-152 and NRP-154 cells were washed twice in cold FACS buffer (PBS/0.1% serum/0.01% NaN_3). Cells were blocked by incubation on ice in goat Ig (Sigma), 2 mg/ml, for 45 min. After washing twice, cells were incubated with 1 or 2 µg/10^6 cells in 100 µl biotinylated goat anti-human IL-6 receptor (ligand-affinity purified; R&D Systems) on ice for 45 min. After washing three times, cells were incubated with phycoerythrin-labeled streptavidin for 45 min on ice. After washing three times, cells were analyzed on the flow cytometer.

**Treatment of NRP-152 and NRP-154 cells with AG490**

The tyrphostin protein kinase inhibitor AG490 was purchased from Calbiochem. It was dissolved in DMSO, and stored at -20°C in single-use aliquots. NRP-152 and cloned NRP-154 cells were placed in 60 mm wells, and treated with AG490 for 48 hrs. The cells were removed with trypsin, and stained after washing with FITC-annexin V (5 µl/10^6 cells; Caltag) for 15 min at room temperature. Apoptotic cells (cells staining with FITC-annexin V) were quantified by measuring green fluorescence in FL1 on the flow cytometer. CellQuest software was used to acquire and analyze the data. STATView software was used to perform statistical analyses.

**Results**

**Determination of the phosphorylation state of STAT3 in NRP-152 & NRP-154 cells**

We had very preliminary data, from a microarray experiment comparing the RNA of NRP-152 to NRP-154 cells, which indicated that STAT3 might be over-expressed on NRP-154 cells relative to NRP-152 cells (data not shown). Since STAT3 is active only when phosphorylated, we decided to confirm these preliminary results by looking for phospho-STAT3 in both cell lines by intracellular flow cytometry. We observed that STAT3 was constitutively phosphorylated in NRP-154, but not NRP-152 cells. Even when treated with testosterone as described in Materials and Methods, NRP-152 cells did not exhibit phosphorylated STAT3 (Figure 1). These data indicate that the over-expressed STAT3 observed in NRP-154 cells in the gene microarray was putatively active as a transcription factor, since the amount of phospho-STAT3 in NRP-154 cells was increased relative to NRP-152 cells.

**IL-6 is produced constitutively by NRP-152 and NRP-154 cells**

In order to determine if IL-6 was the activating ligand for phosphorylation of STAT3, we examined IL-6 expression in untreated NRP-152 and NRP-154 cells by intracellular flow cytometry. While both cell lines made IL-6, NRP-154 cells displayed a different pattern, in that there was a
peak of cells negative for IL-6 (Figure 2). This may have been due to heterogeneity of the NRP-154 cells, since the parental lines had not been subcloned by us. Therefore, we derived 16 clones of NRP-154 cells by limit-dilution cloning, and examined these for level of IL-6 expression. We observed that 9 clones were highly-positive for IL-6, while the rest expressed little IL-6, thereby accounting for the 2 peaks we observed in the parental NRP-154 line (Figure 3).

**IL-6 receptor was expressed on the surfaces of NRP-152 and NRP-154 cells**

When we examined NRP-152 and uncloned NRP-154 cells for expression of the IL-6 receptor, we observed that all NRP-152 cells expressed the IL-6 receptor, while the uncloned NRP-154 cells had a population that apparently did not express the receptor (Figure 4). In studies currently underway, we are analyzing the 16 NRP-154 clones we derived for differences in IL-6 receptor expression, and to see if there is any correlation with levels of IL-6 expression.

**Effect of dexamethasone treatment on NRP-152 and NRP-154 cells**

To examine the role of autocrine IL-6 on NRP-152 and NRP-154 cells, we looked at the effect of dexamethasone treatment on IL-6 expression on both cell lines, and on high and low IL-6-expressing clones of NRP-154 cells. Dexamethasone is known to inhibit IL-6 synthesis by acting at the steroid-response elements of the IL-6 promoter. NRP-152 and NRP-154 cells were routinely grown in the presence of dexamethasone, so the effect on proliferation was not quantified. In the following experi-
ments, we used dexamethasone-free medium, and also treated the serum twice with activated charcoal, to remove endogenous steroids (stripped serum), then added defined amounts of dexamethasone for carrying out the experiments. We observed that dexamethasone treatment of NRP-152 cells inhibited IL-6 synthesis without affecting NRP-152 growth, as expected. In contrast, dexamethasone treatment of NRP-154 cells did not inhibit IL-6 synthesis; instead dexamethasone treatment apparently enhanced IL-6 production (panel B). This effect was observed for high and low IL-6-expressing clones of NRP-154 (Figure 5, panels C and D), and therefore was independent of IL-6 production by the cells. However, clones expressing low amounts of IL-6 expressed less enhancement of IL-6 production that clones expressing high amounts of IL-6 (Figure 5, panels C and D).

**Inhibition of JAK2 induced apoptosis in NRP-152 but not NRP-154 clones**

In continuing our studies on the role of IL-6 in STAT3 phosphorylation, we used the JAK2 phosphorylation inhibitor AG490, to see if inhibiting JAK2 phosphorylation would inhibit survival of NRP-152 or NRP-154 cells, as had been described for LNCaP cells previously [29]. We examined clones of NRP-154, selected by amount of IL-6 expression each had: 2 high IL-6-expressing clones and 2 low-IL-6-expressing clones were used for these experiments. Treatment with the JAK2 inhibitor for 48 hrs induced apoptosis, measured by cellular binding of FITC-annexin V, in NRP-152 but not NRP-154 clones (Table 1). We detected no effect of the vehicle (DMSO) at the highest concentration used. Our data imply that JAK2-mediated phosphorylation of STAT3 was essential for the
survival of NRP-152 cells, but that NRP-154 cells did not require activated JAK2 for survival, regardless of amount of IL-6 produced by the cell. Further experiments are underway to determine if STAT3 requires phosphorylation by a different kinase, such as JAK1, in NRP-154 cells.

Discussion
We observed that STAT3 was constitutively phosphorylated in NRP-154, but not NRP-152 cells (Figure 1). Treatment of NRP-154 cells with testosterone did not increase the level of phosphorylation in NRP-154 cells (data not shown). Even after testosterone treatment, STAT3 was not phosphorylated in NRP-152 cells (data not shown). There is evidence that androgen treatment may increase the survival of PCA cells through activation of STAT3 [29]. Further experiments are underway to test this possibility. STAT1, another signaling intermediate in the IL-6 pathway, has been observed to be activated in non-tumorigenic cells, and may function as a "check" for STAT3 phosphorylation, a possible oncogenic event [30,31]. We are investigating whether or not STAT1 is phosphorylated by JAK2 in NRP-152 cells, and whether STAT1 phosphorylation is required for the survival of

Figure 5
Dexamethasone treatment inhibited IL-6 expression in NRP-152 but not NRP-154 cells. Cells were grown in charcoal-adsorbed serum-containing medium prior to beginning dexamethasone treatment (10^{-6} M) for 2 days. Cells were harvested, fixed, and permeabilized, stained for IL-6, then analyzed for fluorescence in FL2, as described above. Panel A, NRP-152 cells. Panel B, NRP-154 cells. Panel C, NRP-154 clone A3 (high IL-6 expressing). Panel D, NRP-154 clone G7 (low IL-6-expressing). All panels, purple line = no dexamethasone; red line in panels A & B = + dexamethasone; blue line in panels C & D = + dexamethasone.
NRP-152 cells. These results would explain why we saw an effect of AG490 on NRP-152 cells, in the absence of phospho-STAT3 in these cells (Table 1).

Table 1: Inhibition of JAK2 resulted in apoptosis of NRP-152 cells

| Cell        | Rx   | µM | % Apoptotic +/- SD |
|-------------|------|----|-------------------|
| NRP-154     | AG490| 0  | 8 + 4             |
| (uncloned)  |      | 30 | 8.5 ± 5           |
|             |      | 100| 7.5 ± 5           |
| 154 clone A3| AG490| 0  | 7.2 ± 2           |
| (high IL-6) |      | 100| 9.8 ± 3           |
| 154 clone B1| AG490| 0  | 8.5 ± 5           |
| (high IL-6) |      | 100| 9.3 ± 4           |
| 154 clone G3| AG490| 0  | 3.7 ± 3           |
| (low IL-6)  |      | 100| 8.3 ± 5           |
| 154 clone G7| AG490| 0  | 13.2 ± 5          |
| (low IL-6)  |      | 100| 11.9 ± 7          |
| NRP-152     | AG490| 0  | 7.5 ± 4           |
|             |      | 30 | 12 ± 0            |
|             |      | 100| 45 ± 10*          |

Legend: NRP-152 and NRP-154 cells were placed in 60 mm plates for 48 hr with compound at the concentrations indicated. Zero concentration of the compound is the vehicle (DMSO) control. At the end of the incubation period, cells were harvested, washed, and stained with FITC-annexin V, to demonstrate apoptotic cells. Quantification of fluorescence was performed on a Becton-Dickinson FACScan flow cytometer using CellQuest software. * p < 0.005 by Student t-test, compared to vehicle-treated cells.

We looked at the effect of anti-rat IL-6 Ab on both NRP-152 or NRP-154 cell growth rate, but were unable to detect an effect, using 3H-thymidine incorporation to measure proliferation (data not shown). We think this is due to failure to reach high enough Ab (commercially-available anti-rat IL-6) concentrations necessary to neutralize the IL-6 produced by the NRP-154 cells (highest concentration achieved was only 20 µg/ml). We are limited in performing Ab studies by the lack of commercially-available rat-specific reagents. However, we are using alternative strategies in more experiments currently underway to determine the role of IL-6 in STAT3 activation to answer this important question in NRP-154 cells.

We found that dexamethasone treatment of NRP-152 cells inhibited IL-6 synthesis without affecting cell growth. In contrast, dexamethasone treatment of NRP-154 cells did not inhibit IL-6 synthesis; instead dexamethasone treatment eliminated the IL-6 negative peak and enhanced IL-6 production albeit to a smaller extent in low IL-6-expressing clones (Figure 5, panel D). Enhancement of IL-6 production by dexamethasone treatment has been previously observed in Kaposi's sarcoma cells [32]. In the case of NRP-154 cells, the steroid-responsive element for dexamethasone on the IL-6 promoter may have been mutated to a form that does not bind steroid receptors. Mutations in the IL-6 promoter region may play a role in the tumorigenic effects of constitutive IL-6 expression in prostatic carcinoma cells [33]. Such polymorphisms have been described for the IL-6 receptor in Kaposi's sarcoma, and are believed to play a role in IL-6-mediated progression of this type of cancer [32].

We observed that inhibition of STAT3 activation by treatment with AG490, which inhibits JAK2 activation, resulted in apoptosis of NRP-152 but not NRP-154 cells (Table 1). A possible explanation of the data would be the use of JAK1 for phosphorylation of STAT3 in NRP-154 cells, which would not be inhibited by AG490. IL-6 receptor binding activates JAK1 as well as JAK2, which in turn phosphorylates STAT3 [14]. This in fact has been shown to be the case for v-src-transformed fibroblasts [34]. Another hypothesis is that STAT3 activation in NRP-154 cells is not dependent upon a signaling cascade, but is constitutive, possibly due to a mutation not unlike that contained within the cSTAT3 plasmid generated by Bromberg, et al. [35]. We are investigating in detail the signaling pathway in NRP-152 and NRP-154 cells to answer these important questions.

The importance of STAT3 activation via IL-6 in prostatic cancer development has been suggested by previous investigators. For example, IL-6 acting via its receptor has been shown to activate STAT3 in LNCaP cells. IL-6 given exogenously, since LNCaP cells do not produce IL-6, resulted in increased growth of the cells concomitant with activation of STAT3 [36]. LNCaP cells transfected with a plasmid conferring constitutive IL-6 expression demonstrated increased growth, relative to untransfected or sham-transfected cells [36]. However, other investigators have observed that IL-6 treatment of LNCaP cells resulted in terminal differentiation and inhibition of growth, associated with STAT3 activation [17,37,38]. The molecular basis for the apparent contradiction is unknown at this time. Continued use of the NRP-152 and NRP-154 cell lines in parallel experiments should be useful in elucidating the discrepancies among various laboratories.

It is possible that exogenous or autocrine IL-6 is not required for constitutive STAT3 activation in NRP-154 cells. For example, viral IL-6 might be incorporated into the genomes of prostatic carcinomas, as has been described for Kaposi sarcoma [5,32,33,39–41]. The route of introduction of the viral IL-6 is believed to be through previous herpesvirus infection [39,41]. Another possibility is that the insertion of the oncogene BRCA1 results in the constitutive activation of STAT3 in NRP-154 cells, as...
has been described in Du-145 prostate cancer cells [42]. Du-145 cells do not make IL-6; nor are they dependent upon it for continued proliferation in vitro. However, they were dependent upon STAT3 activation for survival, as demonstrated by experiments in which anti-sense oligonucleotides for STAT3 were incorporated by Du-145 cells [42]. We are currently performing similar experiments to determine if IL-6 is a necessary ligand for the activation and survival of NRP-154 cells.

In summary, we have demonstrated that a major difference between NRP-152 and NRP-154 cells is that NRP-154 cells over-express the gene for STAT3, relative to NRP-152 cells, and untreated NRP-154 cells. Furthermore, NRP-154 cells express constitutively-activated STAT3, while NRP-152 cells do not. Moreover, we found that while both cell lines synthesized IL-6 constitutively, only the IL-6 production of NRP-152 cells was inhibited by dexamethasone treatment (Figure 5). Finally, we demonstrated that while both cell lines expressed the IL-6 receptor on their surfaces, the patterns were different. NRP-154 cells had a subpopulation of cells which did not stain with anti-IL-6 receptor Ab, while all the NRP-152 cells stained with Ab to the IL-6 receptor. Although the results presented above give us more insight into the role of IL-6 in PCA, they do not tell us if constitutive STAT3 activation is a determining factor in the change to prostate neoplasia, or if anti-apoptotic factors induced by STAT3 play a role in prostatic neoplasia.

Conclusions
We have shown that NRP-152 and NRP-154 cells exhibit fundamental differences in the regulation of IL-6 production by dexamethasone, and in the requirement for JAK2-mediated events for survival. The tumorigenic line NRP-154 expressed phospho-STAT3 under normal growth condition, while the non-tumorigenic line did not. These data indicate a very important role for STAT3 in conferring the neoplastic state on prostatic epithelial cells, and point out the future direction of our laboratory's investigations.

List of abbreviations
PCA prostate cancer
DMEM Dulbecco’s modification of Eagle’s medium
GAPDH glyceraldehyde phosphate dehydrogenase
IMDM Iscove’s modification of Dulbecco’s medium
PBS phosphate-buffered saline FITC fluorescein isothiocyanate

Competing interests
None declared.

Acknowledgments
We wish to thank Dr. Arnold Barton for critical reading of the manuscript. This work was supported by the Dean’s Biomedical Grant of UMDNJ, C.R. Bard, Endowed funds, and by a veterans’ Administration Rehabilitation Research and Development Service award.

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