Activation of Signal Transducer and Activator of Transcription 5 in Human Prostate Cancer Is Associated with High Histological Grade

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ABSTRACT

We have recently identified signal transducer and activator of transcription 5 (Stat5) as a critical survival factor for prostate cancer cells. We now report that activation of Stat5 is associated with high histological grade of human prostate cancer. Specifically, immunohistochemical analysis demonstrated a strong positive correlation with activation of Stat5 and high Gleason score in 114 human prostate cancers. To investigate the mechanisms underlying constitutive activation of Stat5 in prostate cancer, we examined the ability of Jak2 to activate Stat5 in prostate cancer cells. We found that Jak2 phosphorylates Stat5 and restores constitutive activation of both Jak2 and Stat5 in CWR22Rv human prostate cancer cells and in CWR22Rv xenograft tumors. In addition, Prl restored constitutive activation of Stat5 in human prostate cancer specimens in ex vivo long-term organ cultures. Finally, Prl protein was localized in the epithelium of 54% of 80 human prostate cancer specimens with positive correlation with high Gleason scores and activation of Stat5. In conclusion, our data indicate that Stat5 is primarily active in high-grade prostate cancer. The results further suggest that Jak2 is the principal Stat5 tyrosine kinase in human prostate cancer, possibly activated by autocrine/paracrine Prl.

INTRODUCTION

Androgens, the primary pharmacological treatment of prostate cancer (1), inhibits androgen-dependent proliferation of prostate cancer cells and induces tumor cell apoptosis. However, androgen ablation provides only a transient remission of the disease, because androgen-independent regrowth of prostate cancer is, in most cases, inevitable (1). The molecular mechanisms of androgen-independent growth of prostate cancer are still largely unclear. Androgen-independent proliferation and survival of prostate cancer cells are thought to be mediated by several different mechanisms, such as the following: (a) by increased expression of androgen receptor (AR; Ref. 2); (b) by altered ligand specificity of AR attributable to mutations in AR (3); and (c) by activation of kinase signaling cascades, which stimulate prostate cancer cell growth either independently of AR or by influencing the activation of AR (4–9).

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We have recently identified transcription factor Signal Transducer and Activator of Transcription 5 (Stat5) as a critical survival protein for human prostate cancer cells (4). Stat5 is a latent cytoplasmic protein, which is comprised of two highly homologous isoforms, 94-kDa Stat5a and 92-kDa Stat5b (10). Stat5 becomes activated by phosphorylation of a specific tyrosine residue (11, 12), after which phosphorylated Stat proteins dimerize and translocate to nucleus where they bind to specific response elements of target gene promoters to regulate transcription (11, 12). Stat5 does not possess intrinsic tyrosine kinase activity and, therefore, requires an upstream kinase for activation (13). Members of Janus kinase (Jak)-kinase protein family are known to phosphorylate Stat5 in mammary and hematopoietic cells (11, 13). In addition to being activated by Jak kinases, Stat5 can be activated in some cell types by receptor and nonreceptor tyrosine kinases (14–17) and by tyrosine kinase fusion oncoproteins such as BCR-ABL and Tel-Jak2 (18, 19).

The factors involved in activation of Stat5 in human prostate cancer cells are unclear. In normal rat prostate epithelium, we have shown previously that transcription factor Stat5 is the central signaling protein activated by prolactin (Prl; Ref. 20). Prl, at the same time, is known to inhibit androgen deprivation-induced apoptotic cell death of prostate epithelium (21) and promote proliferation of both normal (21–23) and malignant prostate epithelial cells (24, 25). Receptors for Prl are expressed in human prostate cancer (26), and a Prl antagonist has been reported to inhibit prostate xenograft tumor initiation and growth in nude mice (27). Moreover, Prl-transgenic mice develop significant enlargement of prostate (28–30). However, clinical studies have not revealed differences in serum Prl levels of men with prostate disease compared with age-matched controls (31, 32).

On the basis of our previous findings of a survival function of Stat5 in human prostate cancer cells (4), we hypothesized that activation of Stat5 may be associated with more biologically aggressive behavior of prostate cancer. Furthermore, we wanted to determine the mechanisms underlying continuous activation of Stat5 in prostate cancer cells, because additional molecular targets might prove useful for therapy development for prostate cancer based on inhibition of Stat5. Here, we show that activation of Stat5 strongly correlates with high histological grade of human prostate cancer. We also show that Prl activates Stat5 in human prostate cancer specimens and in CWR22Rv cells both in vitro and in vivo when grown as tumors in nude mice. Both basal and Prl-induced activation of Stat5 in CWR22Rv human prostate cancer cells were blocked by adenosine expression of dominant-negative (Dn) mutant of Jak2, suggesting that Jak2 is the upstream protein kinase that activates Stat5 in human prostate cancer. Finally, we showed that Prl protein was expressed in 54% of 80 human prostate cancers with increased expression in high-grade prostate cancer and correlation with activated Stat5. Collectively, our results indicate that Stat5 is primarily active in high-grade prostate cancers and that Jak2 is the key upstream kinase responsible for activating Stat5 in prostate cancer cells, possibly by autocrine/paracrine Prl.
MATERIALS AND METHODS

Prostate Cancer Samples. For immunohistochemical analysis, we obtained 103 human prostate cancer specimens (Georgetown University Hospital) in formalin (Table 1). The Gleason (GI) grades and the GI score (33) of each sample were verified by a board-certified pathologist (B. S.). For organ cultures, the prostate cancer specimens were obtained from 11 patients (age range 54–69 years; mean age ± SD, 62.5 ± 4.5 years) with localized or locally advanced prostate cancer (Table 2) undergoing radical prostatectomy. The prostate tissues were obtained after informed consent of the patient and the approval of the Ethical Committee of University Hospital of Turku. Within 1–3 h of the surgery, a board-certified pathologist (K. A.) made a selection of the tissue slices of prostate cancer nodules that were to be available for ex vivo organ cultures or to be frozen for other analyses. The selection of the area was assisted by the clinical information of the localization of the cancer based on the location of the needle biopsy taken at the time of diagnosis and by frozen sections. The rest of each prostate was fixed in formalin, and the whole prostate cross-sections were used for histological grading, for confirming that the areas cultured in organ culture were cancer, and for immunohistochemical stainings.

Ex vivo Long-Term Organ Cultures of Human Prostate Cancer. The prostate organ cultures were performed as described previously (20–23, 34–36). The explants were cultured for 7 days in the presence of 100 nM dihydrotestosterone (5α-androstane-17β-ol-3-one, Sigma; Refs. 23 and 34), and on the 7th day of culture, half of the explants were stimulated with 100 nM hPrl (a gift from Dr. A. F. Parlow; The National Pituitary Hormone Program, National Institutes of Diabetes, Digestive and Kidney Diseases; Ref. 20) for 1 h, after which the explants were fixed in formalin. Four parallel dishes each containing 20-prostate cancer explants were cultured in both treatment groups.

Xenograft Tumors and Cell Culture. Castrated male athymic mice were purchased from Taconic (Germantown, NY) and cared for according to the institutional guidelines. Briefly, 5 × 10^6 CWR22Rv cells were mixed with one quarter of the total injection volume of 0.2 ml with Matrigel. Simultaneously with the tumor cell inoculation (2 sites/mouse), sustained-release testosterone pellets (12.5 mg/pellet, 1 pellet/mouse; Innovative Research of America, Sarasota, FL) were implanted s.c. When the tumors (n = 12) reached 12–15 mm in size, half of the mice were injected i.p. with human Prl (hPrl; 3.0 μg/g body weight; n = 3) or PBS (n = 3). The mice were sacrificed, and the tumor tissues were harvested 30 min after the injection.

For immunoprecipitation of Stat5a and Stat5b, CWR22Rv cells were harvested at 50% or 100% confluency. For adenoviral gene delivery, CWR22Rv cells were infected with AdΔNJak2 or AdWTJak2 at multiplicity of infection 10 for 90 min after which RPMI 1640 containing 10% FCS was added. Before the stimulation of CWR22Rv and T47D breast cancer cells with hPrl, the cells were starved in serum-free medium for 16 h (37). For Stat5 immunoprecipitations, cells were incubated for 15 min, and for Jak2 immunoprecipitations, CWR22Rv and T47D cells were incubated for 10 min in the presence or absence of 10 nm hPrl, and the cell pellets were frozen at −70°C or fixed with 4% paraformaldehyde for immunocytochemistry.

Immunohistochemistry. Regular paraffin sections of 103 prostate cancer samples (Table 1), cross-sections of the entire prostate (Fig. 1f; Table 2) of 11 patients with localized or locally advanced prostate cancer, and paraffin sections of cultured explants of human prostate cancer tissues were immunostained for activated Stat5, total Stat5, or Prl. Immunohistochemistry of activated Stat5 was performed as described previously (38). Briefly, tissue sections or parafomaldehyde-fixed cells were microwave-treated with antigen retrieval solution AXAR1 (Advantex BioReagents, Courouc, TX). For the detection of total Stat5, parallel tissue sections were microwave-treated in citrate solution (BioGenex Laboratories, San Ramon, CA). Sections immunostained for Prl were treated with pepsin (2.5 mg/ml; BioGenex) for 10 min at 37°C to unmask the epitopes. Endogeneous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide. The primary antibodies recognizing phosphorylated (Y694/Y699) Stat5 monoclonal antibody (mAb; Advantex BioReagents), total Stat5 (mAb; Santa Cruz Biotechnology, Santa Monica, CA), and Prl (mAb; BioGenex) were diluted in 1% BSA in PBS at concentrations of 0.6 μg/ml, 2 μg/ml, and 1:40, respectively. For immunohistochemical detection of prostate-specific antigen in cultured human prostate cancer explants, a polyclonal antiprstate-specific antigen antibody was used (BioGenex, ready-to-use preparation). Antigen-antibody complexes were detected using appropriate biotinylated goat secondary antibodies followed by streptavidin-horseradish-peroxidase complex (BioGenex). 3,3’-Diaminobenzidine was used as chromogen and hematoxylin was used as counterstain. For controls, subtype-specific mouse IgG or normal rabbit serum was used as appropriate. Lactating human breast tissue (38) was used as a positive control tissue for total and activated Stat5 immunohistochemistry and human pituitary prolactinoma for Prl immunohistochemistry, respectively. For immunohistochemical detection of Prl protein, additional control sections of human prostate cancer were immunostained with anti-hPrl mAb in the presence or absence of 100-fold excess of hPrl.

Scoring of Levels of Active and Nuclear Stat5 in Prostate Cancer Specimens. Individual prostate cancer samples were scored (M. T. N. and H. L.) for active Stat5 and Prl levels and these three levels were used as the basis for increasing staining intensity, where 0 was undetectable, low immunostaining gave 1+, intermediate immunostaining gave 2+, and high immunostaining gave 3+ as a score.

Statistical Analysis. The GI scores of the prostate cancer specimens immunostained for activated Stat5 (114 records) and for Prl protein (80 records) were classified into three groups (4–6, 7, 8–9). Because both the GI score and immunostaining intensity (Stat5P, Prl) are ordinal categorical variables, the Spearman’s rank-order correlation coefficients (39) were calculated, and the test of the correlation coefficient equals zero was performed. All of the analyses were performed using SAS software (40).

Solubilization of Proteins, Immunoprecipitation, and Immunoblotting. Prostate cancer cells and tissues were lysed in lysis buffer (10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium PP, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotonin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). The protein concentrations of clarified tissue lysates were determined by a simplified Bradford method (Bio-Rad Laboratories Inc., Hercules, CA). Depending on the experiment, 4 mg of tissue protein and 1 ml of the cell lysates were used for immunoprecipitation for 3 h at 4°C with polyclonal rabbit antisera against either Stat5a, Stat5b, or Jak2 (2 μl/ml; Advantex Bioreagents). Both Stat5a and Stat5b were simultaneously immunoprecipitated from the human prostate cancer samples. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharzmacia Biotech, Piscataway, NJ).

Table 1 Relationship between active Stat5 and Gleason score Note

<table>
<thead>
<tr>
<th>Activation of Stat5 immunostaining</th>
<th>Gleason score</th>
<th>n</th>
</tr>
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<tr>
<td></td>
<td>4–6</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>+ +</td>
<td>8–9</td>
<td>2</td>
</tr>
<tr>
<td>+ + +</td>
<td>n</td>
<td>16</td>
</tr>
</tbody>
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Note: *Spearman’s rank-order correlation coefficient for active Stat5 versus Gleason score was 0.6088 (P < 0.0001).

Table 2 Prostate cancer samples cultured in organ culture

<table>
<thead>
<tr>
<th>Age at the day of operation</th>
<th>Gleason grade</th>
<th>Gleason score</th>
<th>WHO grade</th>
<th>Stage</th>
<th>Volume % of the tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>54–56</td>
<td>(2 + 2)</td>
<td>4</td>
<td>1</td>
<td>T2a</td>
<td>10–15</td>
</tr>
<tr>
<td>60–63</td>
<td>(2 + 2)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>10–15</td>
</tr>
<tr>
<td>65–69</td>
<td>(2 + 2)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>5–10</td>
</tr>
<tr>
<td>69–73</td>
<td>(2 + 3)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>5–10</td>
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<tr>
<td>69–73</td>
<td>(2 + 3)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>10–15</td>
</tr>
<tr>
<td>69–73</td>
<td>(2 + 3)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>15–20</td>
</tr>
<tr>
<td>69–73</td>
<td>(2 + 3)</td>
<td>5</td>
<td>1</td>
<td>T2a</td>
<td>5–10</td>
</tr>
<tr>
<td>69–73</td>
<td>(2 + 4)</td>
<td>6</td>
<td>1</td>
<td>T2a</td>
<td>10–15</td>
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<tr>
<td>69–73</td>
<td>(2 + 4)</td>
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<td>69–73</td>
<td>(2 + 4)</td>
<td>6</td>
<td>1</td>
<td>T2a</td>
<td>7–80</td>
</tr>
<tr>
<td>69–73</td>
<td>(2 + 4)</td>
<td>6</td>
<td>1</td>
<td>T2a</td>
<td>5–10</td>
</tr>
</tbody>
</table>

Note: *Ages of the patients ranged from 54 to 69 yrs. at the time of the operation (mean age 62.5 ± 4.5 SD yrs).

Stat5 cancers in which Stat5 was activated by Prl in organ culture.

Prostate cancers that had constitutive activation of Stat5 prior to culture.

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Fig. 1. Signal transducer and activator of transcription 5 (Stat5) is active in human prostate cancer. Paraffin-embedded tissue sections of human prostate specimens were immunostained with a monoclonal activation-state-specific anti-phosphorylated Stat5 antibody (B, C, E, G) and with a monoclonal anti-Stat5 antibody (B, D, F, H). 3.3′-diaminobenzidine was used as a chromogen and Mayer hematoxylin as counterstain. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intense positive reactions for active Stat5 in the nuclei of epithelial cells of poorly differentiated prostate cancer acini (Gleason 4 + 5 = 9, patterns 4 and 5 shown; A, B) and moderately differentiated prostate adenocarcinoma (Gleason 3 + 4 = 7, pattern 3 shown; C, D), whereas adjacent normal secretory prostate epithelium was negative for phosphorylated Stat5 (E), and predominant nuclear localization of immunostaining for Stat5 protein was absent (F). Cross-sections of entire prostate (I), cross-sections were taken proximal to verumontanum; arrows point at the ejaculatory ducts) from 11 patients with localized or locally advanced prostate cancer were analyzed by immunohistochemistry for activation of Stat5. Basal epithelial cells of perirethral prostate glands with normal acinar morphology in transition zone stained positively for activated nuclear Stat5 (G, H). Lactating human breast is presented as a positive control (I, J). Lactation positively for activated nuclear Stat5 (G, H). Nuclear localization of Stat5 protein (C, D, F, H) stained with subtype-specific mouse IgG were negative. Stat5a and Stat5b were collectively immunoprecipitated from tissue homogenate (J, K) and moderately differentiated human prostatic adenocarcinoma (K), and parallel sections of lactating human breast (L) were size-separated on 2% Tris-borate EDTA-agarose gels. The glyceraldehyde-3-phosphate dehydrogenase gene was amplified in a separate reaction to confirm the integrity of the first-strand cDNA of each sample, and H2O was used as a negative control. A-H and I-L bar, 15 μm. Arrows, positive nuclear immunostaining for activated Stat5 (A and C), nuclear localization of Stat5 protein (D), and positive immunostaining of basal epithelial cells (G and H).

Samples were subjected to 7.5% SDS-PAGE under reducing conditions. The primary antibodies were used at the following concentrations: anti-phosphotyrosine-Stat5α/β (Y694/Y699) mAb (1 μg/ml; Advantex BioReagents); anti-Stat5a pAb (1:3000; Advantex BioReagents); anti-Stat5b pAb (1:3000; Advantex BioReagents); antiphosphorytrosine mAb (1:1000; Upstate Biotechnology, Lake Placid, NY); anti-Jak2 pAb (1:3000; Upstate Biotechnology); and anti-panStat5 mAb (1:1000, Transduction Laboratories, Inc.). Antigen-antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham, Piscataway, NJ) and exposed to film.

Generation of Adenovirus for Gene Delivery of DnJak2 and Wild-Type Jak2 (WtJak2). Replication-defective human adenovirus (Ad5) carrying Jak2 (WtJak2). Replication-defective human adenovirus (Ad5) carrying DnJak2 or WtJak2 was generated using the AdEasy Vector system (Qbiogene, Carlsbad, CA). Briefly, V5/His epitope-tagged WtJak2 and DnJak2 expression vectors were derived from rat Jak2 cDNA (41), as described previously (42), and were subsequently released by Apal and NotI digestion and subcloned into the NotI and EcoRV sites of Adv-shuttle vector under cytomegalovirus promoter. Before NotI digestion, the Apal digested ends were blunt-ended by T4 DNA polymerase. Homologous recombination of WtJak2 and DnJak2 transfer vectors with the AdEasy vector was performed in BJ5183 Escherichia coli by electroporation. Recombined clones were screened by kanamycin-resistant growth, and the recombinant viruses were packaged in QBI-293 cells. Resulting clones were selected from plaques and amplified. Expression of WtJak2 and DnJak2 from adenoviral stocks was verified by Western blotting using an anti-Jak2 antibody (Upstate Biotechnology). Selected recombinant viral stocks were expanded in large-scale cultures, purified by double-cesium chloride gradient centrifugation, and titered side-by-side by a standard plaque method in QBI-293 cells as per the manufacturer’s instructions.

Reversal Transcription (RT)-PCR. For detection of Stat5a, we used the following primer pair: forward primer (exon 18), 5′-CACAGATCAAGACAG TGTC-3′ and reverse primer (exon 20), 5′-CTGTCCATTTGGTCGCGGTA-3′. The size of the PCR product yielded by this primer pair is 247 bp. For detection of Stat5b, we used 5′-GATTCTCAGGAAAGAATGTT GAGGTGTCCTCCAGATCGAA G-3′ (exon 19) as the reverse primer. The size of the PCR product yielded by this primer pair is 413 bp. Total RNA was isolated from CWR22Rv1, LnCap, and Du145 prostate cancer cells, from fresh human prostate cancer specimens and from T-47D breast cancer cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was reverse transcribed from 1 μg of total RNA with Super-Script II reverse transcriptase (Invitrogen) using oligodeoxynucleotidylc acid primers. The RT reaction was incubated at 42°C for 45 min followed by heat inactivation for 15 min at 70°C. The conditions for PCR for all reactions...
RESULTS

Activation of Stat5 Is Associated with High Histological Grade of Human Prostate Cancer. Activation of Stat5 was evaluated in 114 prostate cancer specimens obtained from patients with localized or locally advanced prostate cancer (Table 1). Prostate carcinomas were diagnosed with Gleason tumor scores (33), which, in our material, ranged from 4 to 9 (Table 1). Activation of Stat5 was analyzed in paraffin-embedded tissues by a highly sensitive in situ detection method, which is based on immunohistochemical detection of phosphorylated Stat5 (Ref. 38; Fig. 1, A, C, E, and G). The specificity of antiphosphoStat5 mouse monoclonal antibody AX-1 to tyrosine-phosphorylated Stat5 in immunohistochemistry and immunoblotting has been validated by peptide competition, inducible phosphorylation studies, site-directed mutagenesis, and Stat5 knock-out model analyses (38). This method was complemented with immunohistochemical detection of nuclear Stat5 protein (Fig. 1, B, D, F, and H). Activation of Stat5 was also shown in human prostate cancer by immunoprecipitation of Stat5 followed by Western blot analysis (Fig. 1M). Expression of Stat5a and Stat5b mRNA in human prostate cancer cell lines and prostate cancer specimens was demonstrated by RT-PCR analysis (Fig. 1N).

The immunostained tissue sections were evaluated using a semi-quantitative scoring method, which was based on the intensity of nuclear phosphorylated Stat5 staining. The data in Table 1 show that the activation of Stat5 has a strong positive correlation (r = 0.6088; P < 0.0001) with the Gleason score of prostate cancer, which is further illustrated in Fig. 2. Specifically, as shown in Fig. 2, the percentage of prostate cancer specimens with high immunostaining intensity for nuclear phosphorylated Stat5 increased as the Gleason score increased, whereas the percentage of prostate cancer specimens with low immunostaining intensity or absence of immunostaining for activated Stat5 decreased as Gleason score increased.

Representative samples of poorly differentiated adenocarcinoma (Gleason score 4 = 5 = 9, patterns 4 and 5 shown; Fig. 1, A and B) and moderately differentiated adenocarcinoma (Gleason score 4 = 3 = 7, pattern 3 shown; Fig. 1, C and D) illustrating Stat5 activation by phosphoryrosine detection (Fig. 1, A and C) or nuclear anti-Stat5 detection (Fig. 1, B and D) are presented. Adjacent normal secretory epithelium was negative for Stat5 phosphoryrosine staining (Fig. 1E), and predominant nuclear localization of Stat5 immunostaining was absent (Fig. 1F). Interestingly, immunohistochemical analysis of the cross-sections of entire prostate of 11 patients with localized or locally advanced prostate cancer (Fig. 1H; Table 2) showed that basal epithelial cells of periurethral prostate glands, in particular in the transition zone (Fig. 1I; arrows point at ejaculatory ducts indicating a cross-section proximal to verumontanum), were positive for activated nuclear Stat5 (Fig. 1, G and H). Lactating human breast epithelium is presented as positive control for immunostaining of tyrosine phosphorylated, activated Stat5 (Ref. 38; Fig. 1J), and parallel sections of lactating human breast and human prostate cancer specimens (Fig. 1, K and L, respectively) stained with subtype-specific mouse IgG were negative. We conclude that activation of Stat5 was positively associated with high Gleason score of human prostate cancer.

Prl Activates Stat5 in Androgen-Independent CWR22Rv Cells and in CWR22Rv Xenograft Tumors Grown in Nude Mice. The fact that transcription factor Stat5 is the key signaling protein activated by Prl in normal rat prostate epithelium prompted the hypothesis that Stat5 is activated by Prl also in human prostate cancer. First, we tested the hypothesis in CWR22Rv cells grown in vitro (Fig. 3, A and B). CWR22Rv is an androgen-independent human prostate cancer cell line that has been established from one of the hormone-refractory CWR22R tumors (43). The original tumor was derived from a Gleason score 9 primary prostate cancer with osseous metastases (44), and the primary CWR22 prostate tumor is highly responsive to androgen deprivation with marked tumor regression after castration, mimicking the course of human disease (45). After androgen deprivation-induced regression of the original tumor, the recurred tumors (CWR22R) are not dependent on androgens and are able to grow in female or castrated male mice (45).

In the preliminary experiments, we showed that Prl induced nuclear immunostaining of activated Stat5 in CWR22Rv cells by using activation-state-specific anti-pTyrStat5 antibody (Fig. 3A). To study whether activation of Stat5 induced by Prl was attributable to activation of Stat5a and/or Stat5b, Stat5a and Stat5b were immunoprecipitated from Prl-stimulated and unstimulated exponentially growing (low density) or confluent (high density) CWR22Rv cells and immunoblotted with the anti-pTyrStat5 antibody (Fig. 3B). In exponentially growing CWR22Rv cells, Prl activated both Stat5a and Stat5b (Fig. 3B), whereas in confluent CWR22Rv cells, only phosphorylation of Stat5b, and not Stat5a, was induced by Prl (Fig. 3B). Reblotting of the membranes with corresponding Stat5a and Stat5b antibodies verified equal levels of Stat5 protein loaded per lane (Fig. 3B).

After showing that Prl activates Stat5 in CWR22Rv cell line, we next examined whether Prl activates Stat5 in CWR22Rv cells grown as s.c. tumors in nude mice. Male mice carrying CWR22Rv prostate tumors were injected i.p. with hPrl or vehicle for 30 min. Nuclear immunostaining for activated Stat5 sharply increased in the epithelial cells of CWR22Rv tumors of Prl-treated mice (Fig. 3C). On the basis of these experiments, we concluded that Prl activates Stat5 in CWR22Rv human prostate cancer cells in vitro and in vivo when grown as tumors in nude mice.

Stat5 Is Activated by Prl in ex Vivo Long-Term Organ Cultures of Human Prostate Cancer Specimens. Because Stat5 is the key signal transduction protein activated by Prl in rat prostate epithelium and because Prl activated Stat5 in androgen-independent CWR22Rv prostate cancer cells in vitro and in CWR22Rv cells grown as tumors...
in nude mice, we next wanted to determine whether Prl activates Stat5 also in human prostate cancer samples. To address this question, ex vivo long-term organ cultures of human prostate cancer specimens were established (Fig. 4), as shown previously (23, 34). We have demonstrated the use of organ cultures of normal and malignant human prostate for studies of hormonal regulation of prostate epithelium (23, 34), and recently we demonstrated the use of organ culture of rat prostate as an experimental model for identification of specific intracellular signaling proteins in prostate epithelium activated by hormones and growth factors (20).

Here, we cultured 11 human prostate cancer samples with G1 scores ranging from 4 to 7 (Table 2) for 7 days in organ culture. At the end of each culture, half of the explants were stimulated with 100 nM hPrl for 1 h (Fig. 4). Activation of Stat5 was analyzed by immunohistochemical staining of cultured human prostate cancer explants with monoclonal activation state-specific antibody for Stat5. Also, parallel sections were immunostained for Stat5 protein (data not shown).

Stat5 was constitutively activated in 6 of 11 prostate cancer specimens before organ culture (Table 2). In five of those six prostate cancer samples, Prl restored activation of Stat5 in prostate cancer acini of prostate tissue explants when cultured in low serum conditions in organ culture (Table 2). Representative prostate cancer samples illustrating activation of Stat5 by Prl within malignant epithelium of cultured prostate explants are presented (Fig. 4, A and B). In addition, a prostate cancer specimen that did not respond to Prl stimulation by activation of Stat5 is shown in Fig. 4C. Development of squamous metaplasia in prostate cancer acini of human prostate cancer explants cultured in organ culture was often noted. All prostate cancer samples cultured in organ culture were positive for prostate-specific antigen immunostaining (data not shown).

Fig. 3. Prl activates Stat5 in androgen-independent CWR22Rv prostate cancer cells and in CWR22Rv xenograft tumors grown in athymic mice. A, androgen-independent CWR22Rv cells were cultured exponentially, starved in serum-free medium for 16 h, and incubated with (+Prl) or without (−Prl) human (h)Prl (10 nM) for 15 min and fixed in 4% paraformaldehyde. Cells were immunostained with anti-ρTyStat5a as described in the legend to Fig. 1. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intensive positive nuclear immunostaining in CWR22Rv cells stimulated with Prl (arrow). Prl, prolactin. B, Prl activates both Stat5a and Stat5b in exponentially growing CWR22Rv prostate cancer cells. Stat5a and Stat5b were immunoprecipitated from CWR22Rv cells grown at low (LD; 50% confluency) or at high density (HD; 100% confluency), starved in serum-free medium for 16 h, incubated with (+) or without (−) human Prl (10 nM) for 15 min, resolved on SDS-PAGE, and blotted with a monoclonal anti-ρTyStat5 antibody (aStat5pY, B, top panel and the third panel from the top). Filters were stripped and reblotted with polyclonal antisera against Stat5a (B, the second panel from the top) or Stat5b (B, bottom panel). C, Prl activated Stat5 in CWR22Rv prostate cancer cells grown as xenograft tumors in nude mice. CWR22Rv cells were grown as s.c. tumors (n = 12) in castrated athymic male mice that had been supplied with testosterone implants. Mice received i.p. injection with human Prl (0.3 μg/g body weight) or with vehicle (PBS) for 30 min and sacrificed, and paraffin-embedded tissue sections of tumors were immunostained with a monoclonal anti-ρTyStat5 antibody as described in the legend to Fig. 1. Arrows indicate intense positive immunostaining for active Stat5 in CWR22Rv tumor cells of Prl-treated mice. A and C, bar 20 μm.

Fig. 4. Prl activates Stat5 in ex vivo long-term organ cultures of human prostate cancer. To test whether Prl activates Stat5 in prostate cancer, 11 localized or locally advanced human prostate cancers (Table 2) were cultured for 7 days in organ culture in the presence of androgens (100 nM dihydrotestosterone). At the end of each organ culture, half of the explants were stimulated with human Prl (100 nM) for 1 h. Paraform-embedded tissue sections of cultured prostate cancer explants were immunostained with a monoclonal anti-ρTyStat5 antibody. The immunohistochemistry was performed as described in the legend to Fig. 1. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intensive positive nuclear immunostaining in the epithelial cells of Prl-stimulated explants of human prostate cancer (A and B, +Prl, arrows) compared with epithelial cells of unstimulated explants (A and B, −Prl). A representative prostate cancer that did not respond to Prl stimulation by activation of Stat5 is presented in C. B, bar, 10 μm. A and C, bar, 25 μm.
Activation of Stat5 in Prostate Cancer Cells Is Blocked by Inhibition of Jak2. To identify the tyrosine kinase that phosphorylates Stat5 in human prostate cancer, we hypothesized that Jak2 is the upstream kinase that activates Stat5 in prostate cancer cells. To directly test whether Jak2 is required for phosphorylation of Stat5 in human prostate cancer cells, a DJak2 or WtJak2 was introduced into exponentially growing CWR22Rv prostate cancer cells (Fig. 5A). To determine whether Jak2 also mediated Stat5 activation induced by Prl, we first showed that Prl stimulation of CWR22Rv cells activates Jak2 (Fig. 5B). Next, CWR22Rv cells were mock-infected or infected with adenovirus carrying Djak2 or WtJak2 at multiplicity of infection 10, and 24 h later, cells were starved in serum-free medium for 16 h and then stimulated with Prl. Prl-induced phosphorylation of Stat5 was completely inhibited by Djak2 (Fig. 5C). Furthermore, activation of Stat5 was significantly increased in both exponentially growing and quiescent CWR22Rv cells overexpressing WtJak2 (Fig. 5, A and C). On the basis of these three sets of experiments, we conclude that Jak2 phosphorylates transcription factor Stat5 in CWR22Rv human prostate cancer cells. RT-PCR analysis of prostate cancer cells (CWR22Rv, LnCaP, and DU145) and xenograft tumors showed no evidence for TEL-Jak2 or BCR-ABL chromosomal translocations, which are known to cause constitutive activation of Stat5 in leukemia cells (data not shown).

Prl Is Locally Produced in the Epithelial Cells of Human Prostate Cancer. Because phosphorylation of Stat5 by Prl in prostate cancer cells and in human prostate cancer samples suggested a ligand-induced mechanism for Stat5 activation in human prostate cancer, we hypothesized that Prl is locally produced by the epithelial cells of human prostate cancer. We have shown previously, at both mRNA and protein level, that Prl is locally produced in the epithelium of normal rat (36) and human (23) prostate. In this study, we used immunohistochemical detection of Prl to examine Prl protein expression in human prostate cancer samples of different Gl. scores.

Specifically, we analyzed Prl protein expression in 80 human prostate cancer specimens of different Gl. scores and evaluated the immunostaining with the same semi-quantitative scoring method as was used for the analysis of immunohistochemistry of activated Stat5. A mAb specific to hPrl showed strong immunostaining for Prl in normal human prostate epithelium (Fig. 6A) and in the cytoplasm of the epithelial cells (Fig. 6, B and C) of 54% of 80 prostate cancers analyzed (Table 3). In addition to mAb against Prl, highly corresponding results were obtained by using a polyclonal anti-Prl antibody (data not shown). The highest expression of Prl protein was observed in Gl. score 7, 8, and 9 prostate cancers (Fig. 7; Table 3). Specifically, the intensity of immunostaining for Prl showed significant positive correlation with the Gl. scores (Spearman’s rank-order correlation coefficient 0.3297, P = 0.003).

Moreover, comparison of prostate cancer specimens positive for activated Stat5 with prostate cancer specimens expressing Prl protein also revealed a positive correlation between immunostaining for activated Stat5 and for Prl protein (Spearman’s rank-order correlation coefficient 0.2680, P = 0.0162; Table 4).

In addition to human prostate cancer samples, approximately 20–30% of the epithelial cells of both primary (Fig. 6D) and recurrent (data not shown) CWR22 prostate cancer xenografts grown in nude mice showed positive immunostaining for Prl. Human pituitary prolactinomas were used as positive control tissues (Fig. 6D), and immunostaining of parallel sections with a subtype-specific mouse IgG

Stat5 IS ACTIVATED IN HIGH-GRADE PROSTATE CANCER

Fig. 5. Inhibition of Janus kinase 2 (Jak2) blocks activation of Stat5 in CWR22Rv prostate cancer cells. A, adenoviral expression of dominant-negative Jak2 (DnJak2) blocks activation of Stat5 in exponentially growing CWR22Rv prostate cancer cells. Exponentially growing CWR22Rv cells were mock infected or infected with adenovirus carrying DnJak2 or wild-type Jak2 (WtJak2) at multiplicity of infection 10, and 24 h later, cells were harvested for immunocytochemistry performed as described in the legend to Fig. 1. B, Prl induces activation of Jak2 in CWR22Rv prostate cancer cells. Confluent CWR22Rv prostate cancer cells were starved in serum-free medium for 16 h, incubated with (+) or without (−) human Prl (10 nm) for 10 min, resolved on SDSPAGE, and blotted with a monoclonal anti-pTyr antibody (αpY) with T47D breast cancer cells as positive control (top panel). Filters were stripped and rebotted with a polyclonal anti-Jak2 antiserum (αJak2; bottom panel). C, adenoviral delivery of DnJak2 blocks Prl-induced activation of Stat5 in CWR22Rv cells. CWR22Rv cells were mock infected or infected with adenovirus carrying DnJak2 or WtJak2 at multiplicity of infection 10, and 24 h later, cells were stained in serum-free medium for 16 h, stimulated with human Prl (10 nm) for 15 min, and harvested for immunocytochemistry performed as described in the legend to Fig. 1. A and C, bar 15 μm.

were negative (Fig. 6F). Furthermore, preincubation of anti-Prl antibody with a 100-fold excess of hPrl blocked the immunostaining for Prl in human prostate cancer cells showing the specificity of the immunostaining reaction (Fig. 6, G and H). Thus, we conclude that Prl protein is expressed in the acinar cells of human prostate cancer with increased expression in high-grade prostate cancers.

DISCUSSION

In the present work, we show that activation of transcription factor Stat5 is associated with a high histological grade of human prostate...
cancer. Also, we demonstrate that both Jak2 and Prl are components of a signaling pathway that leads to activation of Stat5 in a large fraction of human prostate cancers. The rationale behind this work centers on our recent identification of Stat5 as a critical survival factor for prostate cancer cells (4).

The prevalent detection of activated Stat5 in high-grade human prostate cancer specimens indicates that Stat5 could be a candidate therapeutic target protein, specifically in advanced prostate cancer. This is of importance for the development of new therapies for prostate cancer, because there are only a few treatment options currently available, particularly for advanced prostate cancer. Positive association of activation of Stat5 and high Gleason grade of prostate cancer raises a question of possible connection of Stat5 with prognosis of prostate cancer. Ongoing work in our laboratory seeks to investigate whether detection of activation of Stat5 in a prostate cancer specimen improves the predictive value of Gleason score in identification of prostate cancer patients who would need more aggressive intervention.

Our finding of preferential activation of Stat5 in high-grade prostate cancers suggests that activation of Stat5 could be one of the molecular mechanisms involved in progression of human prostate cancer. Activation of Stat5 in prostate cancer cells could represent a gain of function event, which may contribute to development of prostate cancer cell clones that are able to grow independently of androgens. In addition to affecting gene transcription directly, Stat5 might interact with androgen receptor and influence its activation in prostate cancer cells, as has been shown for Stat5 interaction with glucocorticoid receptor in mammary epithelial cells (46). As well as being potentially involved in androgen-independent growth of prostate cancer, Stat5 might also contribute to invasion of prostate cancer. On the basis of the present observations, evaluation of Stat5 target genes in

Table 3 Relationship between prolactin immunostaining and Gleason score a

<table>
<thead>
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<th>Prolactin immunostainingb</th>
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a Spearman’s rank-order correlation coefficient for prolactin immunostaining versus Gleason score was 0.3297 (P = 0.003).
b Absence of immunostaining had a score 0, weak positive immunostaining had a score 1+, intermediate had a score 2+, and strong staining intensity had a score 3+.

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**Fig. 6.** Prolactin protein is locally expressed in the epithelial cells of human prostate cancer. Paraffin-embedded sections of prostate cancer from 80 patients and from CWR22 human prostate cancer xenografts grown in nude mice were immunostained with a monoclonal anti-hPrl antibody. Cytoplasm from a number of epithelial cells (arrows) in normal human prostate acini (A) and in prostate cancer acini (B, Gleason pattern 3; C, Gleason pattern 4) showed intense positive immunostaining for Prl protein. Also, epithelial cells of CWR prostate tumors grown in nude mice stained positive for Prl protein (D). Human pituitary prolactinomas were immunostained for Prl as positive control tissues (E), and parallel sections stained with subtype-specific mouse IgG were negative (F). Immunohistochemistry of sections of human prostate cancer with anti-hPrl monoclonal antibody in the absence (G) and in the presence of 100-fold excess of hPrl (H). Excess of hPrl blocked the positive immunostaining for Prl in human prostate cancer cells. A and D, bar, 10 μm and B, C, E, F, G, and H, bar, 20 μm. h, human; Prl, prolactin.

**Fig. 7.** Immunostaining for Prl was associated with high Gleason (Gl.) scores of prostate cancer samples. The Gl. scores of the prostate cancer specimens immunostained for Prl (80 records) were classified into three groups (Gleason scores 4–6, 7, and 8–9). Individual prostate cancer samples were scored for Prl protein immunostaining at three semi-quantitative steps of increasing staining intensity, where 0 was undetectable, low immunostaining gave 1+ intermediate immunostaining gave 2+, and high immunostaining gave 3+ as a score. Prl, prolactin.
prostate cancer will be important, because they are likely to have further therapeutic implications.

A recent study by Gao et al. (47) indicated that in human prostate tissue homogenates there was only a low level of Stat5 binding to an oligonucleotide probe corresponding to the Stat5-response element of the rat β-casein gene promoter in electrophoretic mobility shift assays. In this work we show, by both immunohistochemical staining of phosphorylated nuclear Stat5 protein and by immunoblotting of phosphorylated Stat5, that Stat5 is in the activated state in human prostate cancer. It is possible that when whole tissue homogenates are used in in situ

Table 4 Relationship between active Stat5 immunostaining and prolactin (Prl) protein expression.

<table>
<thead>
<tr>
<th>Active Stat5</th>
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* Spearman’s rank-order correlation coefficient for immunostaining for active Stat5 versus prolactin protein was 0.2680 (P = 0.0162).

Because activation of Stat5 was increased in high-grade human prostate cancers, it would be interesting to determine whether increased activation of Stat5 is attributable to alteration of the level of Stat5 protein itself in malignant prostate epithelial cells. However, the protein levels of Stat5 in different Gl grade prostate cancers would need to be analyzed by immunoprecipitation and immunoblotting of fresh prostate cancer specimens, because immunohistochemical determination of total Stat5 protein levels in prostate epithelium is difficult if Stat5 is not activated and located in the nucleus. As illustrated in Fig. 1F, immunohistochemical detection of nonactivated Stat5 protein appears as diffuse and weak immunostaining in both cytoplasm and nuclei of prostate epithelial cells, which is complicated to quantify reliably. Interestingly, in contrast to secretory epithelium, basal epithelial cells of normal prostate epithelium showed intense activation and nuclear localization of Stat5, particularly in periurethral prostate glands. The basal epithelial compartment of prostate acini has been shown to contain a population of pluripotent progenitor cells that proliferate and differentiate into mature basal and secretory cells (48). We do not know what the role of activation of Stat5 in basal prostate epithelial cells is.

A ligand-induced mechanism for activation of Stat5 in prostate cancer was suggested by the fact that Prl stimulated activation of both Jak2 and Stat5 in CWR22Rv human prostate cancer cells and in CWR22Rv tumors grown in nude mice. Inhibition of Stat5 activation by adenoviral overexpression of Jak2 verified Jak2 as the principal tyrosine kinase that activates Stat5 in CWR22Rv human prostate cancer cells. We also analyzed Prl-induced activation of Stat5 in human prostate cancer samples using organ culture as an experimental model. Prostate organ culture is an in vitro culture system for fresh prostate tissue samples for studies on hormone regulation and signaling in normal and malignant prostate tissue (20–23, 34–36). The principal difference of this model system and cell cultures of primary prostate epithelial cells or cancer cell lines is that in prostate organ culture the whole tissue architecture with critical interactions of epithelium and stroma are maintained. Specifically, we cultured 11 prostate cancer specimens of Gl. scores 4–7 in organ culture (Table 2). In 6 of 11 prostate cancer samples, Stat5 was constitutively activated before culture. Prl restored activation of Stat5 in five of six of these prostate cancer specimens in organ culture, which has low serum conditions. Collectively, these data suggest that Prl-induced activation of Stat5 is one of the mechanisms responsible for Stat5 activation in human prostate cancer.

A study by Leav et al. (26) evaluating Prl-receptor expression in developing normal human prostate and in prostate neoplasia reported similar expression levels of Prl receptors in Gl. grade 3 and 4 prostate cancers with foci of cells with diminished Prl-receptor expression in Gl. grade 4 cancers. In our material, there were moderate regional variations in Stat5 activation in Gl. grade 4 prostate cancers. However, the general level of Stat5 activation was clearly increased in Gl. grade 4 cancers compared with grade 3 prostate cancers. In Gl. grade 5 prostate cancers, Stat5 was highly activated, and the pattern of Stat5 activation was uniform. It is important to note that the material of the study reported by Leav et al. (26) included only a limited number of prostate cancer specimens of Gl. grade 4, and moreover, their material did not contain prostate cancer specimens of Gl. grade 5.

The role of Prl as one of the key ligands that activate Stat5 in human prostate cancer was further supported by the finding of local expression of Prl protein in 54% of prostate cancer specimens and positive correlation with Gl. scores of prostate cancer samples. In addition, expression of Prl protein in prostate cancer specimens correlated positively with activation of Stat5. Previously, using in situ hybridization, Northern blotting, and RT-PCR, we have shown high expression of Prl mRNA in both normal rat and human prostate epithelium (23, 36). Prl has been shown in numerous in vitro studies and mouse model systems to promote prostate epithelial growth (20–24, 26–30, 35, 49, 50). Neutralizing anti-Prl-receptor antibodies may provide a therapeutic approach to inhibit Jak2-Stat5 signaling pathway in human prostate cancer. Moreover, identification of Jak2 as the major kinase that phosphorylates Stat5 in prostate cancer cells provides an alternative approach to block Stat5 in human prostate cancer by small molecule inhibitors of Jak2.

In conclusion, positive correlation of activation of Stat5 with the histological grade of prostate cancer suggests that activation of Stat5 is associated with biologically aggressive behavior of prostate cancer. Prl and tyrosine kinase Jak2 were identified as components of a signaling cascade that activates Stat5 in human prostate cancer cells, and thus, they are potential molecular targets for pharmacological intervention.

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REFERENCES


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Activation of Signal Transducer and Activator of Transcription 5 in Human Prostate Cancer Is Associated with High Histological Grade

Hongzhen Li, Tommi J. Ahonen, Kalle Alanen, et al.


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