Down-regulation of Suppressor of Cytokine Signaling-3 Causes Prostate Cancer Cell Death through Activation of the Extrinsic and Intrinsic Apoptosis Pathways

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Abstract

Suppressor of cytokine signaling-3 (SOCS-3) acts as a negative feedback regulator of the Janus-activated kinase/signal transducers and activators of transcription factors signaling pathway and plays an important role in the development and progression of various cancers. To better understand the role of SOCS-3 in prostate cancer, SOCS-3 expression was down-regulated in DU-145, LNCaP-IL-6+, and PC3 cells by consecutive SOCS-3 small interfering RNA transfections. SOCS-3 mRNA and protein expression as measured by quantitative reverse transcription-PCR and Western blot, respectively, were decreased by ~70% to 80% compared with controls. We observed a significant decrease in cell proliferation and viability in all SOCS-3-positive cell lines but not in the parental LNCaP cell line, which is SOCS-3 negative. In this study, we show that down-regulation of SOCS-3 leads to an increased cell death in prostate cancer cell lines. We found a considerable increase in the activation of the proapoptotic caspase-3/caspase-7, caspase-8, and caspase-9. A significant up-regulation of cleaved poly(ADP-ribose) polymerase and inhibition of Bcl-2 expression was observed in all SOCS-3-positive cell lines. Overexpression of Bcl-2 could rescue cells with decreased SOCS-3 levels from going into apoptosis. Tissue microarray data prove that SOCS-3 is highly expressed in castration-refractory tumor tissue. In conclusion, we show that SOCS-3 is an important protein in the survival machinery in prostate samples. In this study, we show that down-regulation of SOCS-3 results in reactivation of IL-6-induced STAT3 phosphorylation, suggesting an inactivation of the JAK-STAT pathway (22). Overexpression of SOCS-3 differentially affects proliferation of prostate cancer cells, depending on androgen sensitivity (22, 23). However, effects of SOCS-3 down-regulation on apoptosis have not been investigated in detail yet.

Introduction

Dysregulation of the pathway of signal transducer and activator of transcription (STAT) factors has been implicated in various diseases. In many types of carcinomas, the oncogenic role of STAT factors, especially of STAT3, is well documented (1–3). Its overexpression and constitutive activation are correlated with an increased proliferation and metastatic progression. However, in prostate cancer the role of STAT3 is a subject of investigation because its activation is associated with either inhibition or promotion of tumor growth (4, 5). To explain these controversial results, negative feedback regulators of the Janus-activated kinase (JAK)–STAT pathway, especially suppressors of cytokine signaling (SOCSs), have become a focus of interest. The SOCS family consists of eight members, SOCS-1 to SOCS-7 and cytokine-inducible SH2 protein. All these proteins contain a Nterminal region of variable length, a central SH2 domain, and a conserved COOH terminal region called SOCS box. This region interacts with Elongin-B, Elongin-C, Cullin-5, and RING-box-2. The complex can recruit E2 ubiquitin transferases (6). Thus, the proteins can act as E3 ubiquitin ligases and mediate the proteasomal degradation of associated proteins. SOCS-1 and SOCS-3 are especially induced by interleukin-6 (IL-6). IL-6 is a multifunctional cytokine that plays an important role in chronic inflammation of the prostate (7, 8) and in prostate carcinogenesis (9). SOCS-1 and SOCS-3 can inhibit IL-6-mediated JAK activation directly through their kinase inhibitory region as evidenced for the first time in mouse BF-ER cells (10). In addition, it was shown in renal cancer cells that SOCS-3 can also bind with high affinity to phosphotyrosine 757 within the IL-6 receptor subunit glycoprotein 130 (11). It is generally accepted that SOCS-3 acts as an important negative feedback regulator of the IL-6-JAK-STAT signaling pathway (12–14) and plays a crucial role in many biological processes. SOCS-3-deficient mice die as a result of placental defects, whereas enforced expression of SOCS-3 also results in embryonic death (15–17). SOCS-3 is involved in the development and progression of several malignancies. There are indications that SOCS-3 has different functions depending on the tumor origin. In human lung (18), hepatocellular (19), and head and neck cancer (20), SOCS-3 is silenced by hypermethylation, which causes a growth advantage for cancer cells. In contrast, SOCS-3 is detectable in breast cancer (21). In previous studies, we showed that SOCS-3 is expressed in prostate cancer and its expression is inversely correlated with STAT3 phosphorylation, suggesting an inactivation of the JAK-STAT pathway (22). Overexpression of SOCS-3 differentially affects proliferation of prostate cancer cells, depending on androgen sensitivity (22, 23).
poly(AD-ribose) polymerase (cPARP), and a significant decrease of Bcl-2. Immunohistochemical staining proves that SOCS-3 expression increases in castration-refractory prostate cancer (CRPC). Thus, in the present manuscript, we show that SOCS-3 interference with apoptotic pathways is relevant to prostate cancer development and progression.

Materials and Methods

Cell culture and chemicals. LNCaP American Type Culture Collection (ATCC), DU-145, and PC3 prostate cancer cells were obtained from American Type Culture Collection. The LNCaP subline LNCaP-IL-6 was derived in the presence of IL-6, as described elsewhere (24). VCaP cells were a kind gift from Dr. Kenneth Pienta (University of Michigan). All cell lines were cultured in RPMI 1640 (HyClone). Media were supplemented with 10% FCS and 1% penicillin/streptomycin and glutamin.

Short interfering RNA transfection. The short interfering RNA (siRNA) sequence used for targeting human SOCS-3 was previously published (ref. 25; sense 5′-CCAGAACCUGACAGUGACUdTdT-3′, antisense, 5′-UUGAGUCGU-CAGGGUCUUGdtdTdT-3′) and synthesized by Biomers. A nontargeting siRNA pool (Dharmacon) was used as a negative control. siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer’s protocol. All cell lines were transfected with either 2 nmol/L of siRNA against SOCS-3 or control siRNA during a period of 6 d. Two days after the first transfection, the cells were seeded for [3H]thymidine incorporation assay, Western blots, flow cytometry, or caspase assay, respectively. The second transfection was done with the same amount of siRNAs.

RNA isolation and reverse transcription-PCR. Cells were grown on six-well plates. Subsequently, total RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer’s protocol. cDNA synthesis was performed using iScript select cDNA synthesis kit (Bio-Rad). Real-time PCR conditions were as follows: one cycle of denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All amplifications were performed in triplicates. TATA-Box binding protein (TBP) was chosen as an endogenous expression standard (26). Primer and probe sequences are as follows: SOCS-3 forward 5′-TGATCCGCGACAGCTCG-3′, reverse 5′-TCCCAGACTGGGTCTTGACG-3′, TaqMan probe 5′-FAM-CCAGCGCATGCTAGTTC-3′, and a Superblock (IDST1007, IDetect Superstain System HRP, ID Labs.) blocking with an Avidin/Biotin Blocking kit (SP-2001, Vector Laboratories) containing 3% hydrogen peroxidase in PBS. The pretreated slides were antigen retrieval, the slides were microwaved for 7 min in 0.01 mol/L citrate buffer (pH 6) and then allowed to cool for 30 min. After washing with PBS (pH 7.4), the endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide in PBS. In the pretreated slides were blocked with an Avidin/Biotin Blocking kit (SP-2001, Vector Laboratories) and a Super block (IDST1007, IDetect Superstain System HRP, ID Labs.) between washing steps. The primary anti–SOCS-3 antibody (Santa Cruz) was diluted 1:300 in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. For Bcl-2 immunohistochemistry, an antibody from DAKO (M0887) was used at a dilution of 1:80 and staining was performed after microwave treatment in citrate buffer. On the next day, the slides were washed with PBS, and the I Detect Superstain System HRP was used for immunodetection. The substrate detection was performed using the AEC Chromogen/Substrate kit (BP1108, ID Labs.). Slides were counterstained in Mayer’s hematoxylin, cleared, and coverslipped. The percentage

Flow cytometry. DU-145, LNCaP-IL-6+, and PC3 cells were seeded 2 d after the first transfection at a density of 2.5 × 10^4 per well and LNCaP cells were seeded at a density of 3.5 × 10^4 per well onto six-well plates. On the next day, the cells were treated with 2 nmol/L of siRNA against SOCS-3 or with negative control siRNA for 72 h. 25 μL/well of [3H]thymidine (1 μCi/well) were added, and DNA was harvested on 96-well filter plates (UniFilter, Perkin-Elmer) after a 24-h incubation. Scintillation fluid (50 μL) was added, radioactivity was quantified using Chameleon 5025 liquid scintillation counter (HVD Life Sciences).

Caspase assay. For the measurement of the caspase-3/caspase-7, caspase-8, and caspase-9 activity, DU-145 and LNCaP-IL-6+ cells were seeded 2 d after the first transfection at a density of 3.5 × 10^5 per well and LNCaP cells were seeded at a density of 5 × 10^5 per well in triplicates onto 96-well plates. On the next day, the cells were treated with 2 nmol/L of siRNA against SOCS-3 or with negative control siRNA for 72 h. On day 6, assays for caspase activity were performed with the respective Caspase-Glo assay kits (Promega) according to the manufacturer’s protocols. PC3 cells were not used in those experiments because of excessive cell death.

Patient samples. We established tissue microarrays (TMA) using samples from prostate cancer patients comparing normal, prostate intraepithelial neoplasia (PIN), and cancer areas with Gleason scores ranging from 6 to 8. From each patient, two different samples of normal, two samples of high-grade PIN, and four samples of prostate cancer were available. To compare the expression levels from patients without prostate cancer, normal prostate tissue was included from prostate patients who underwent cystoprostatectomy. We introduced four tissue arrays of eight prostate cancer patients. Additionally, six samples were from CRPC. All those patients have received therapy with luteinizing hormone-releasing hormone antagonists. In three cases, docetaxel was given after failure of hormonal therapy.

Immunohistochemistry and TMA. TMAs were assembled using a manual tissue puncher. The different samples were punched out from selected regions of donor tissue and assembled into a new paraffin block. Tissue cores were 2 mm in diameter, and the length ranged from 4 to 6 mm. TMAs were sectioned (4 μm) and stained with H&E to verify histology. The immunohistochemistry stainings were performed on 4-μm sections. For the antigen retrieval, the slides were microwaved for 7 min in 0.01 mol/L citrate buffer (pH 6) and then allowed to cool for 30 min. After washing with PBS (pH 7.4), the endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide in PBS. In the pretreated slides were blocked with an Avidin/Biotin Blocking kit (SP-2001, Vector Laboratories) and a Super block (IDST1007, IDetect Superstain System HRP, ID Labs.) between washing steps. The primary anti–SOCS-3 antibody (Santa Cruz) was diluted 1:300 in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. For Bcl-2 immunochemistry, an antibody from DAKO (M0887) was used at a dilution of 1:80 and staining was performed after microwave treatment in citrate buffer. On the next day, the slides were washed with PBS, and the IDetect Superstain System HRP was used for immunodetection. The substrate detection was performed using the AEC Chromogen/Substrate kit (BP1108, ID Labs.). Slides were counterstained in Mayer’s hematoxylin, cleared, and coverslipped. The percentage
of SOCS-3–positive cells was determined. Pictures were acquired using Tissuequest software (TissueGnostics). The percentage of SOCS-3–positive cells was determined using Histoquest software (TissueGnostics).

**Statistical analysis.** For each treatment group, statistical distribution was determined using Kolmogorov-Smirnov test. Because of non-Gaussian distribution, nonparametric tests were applied as follows. To assess the overall significance for experiments with more than one treatment group, we used the Kruskal-Wallis test. To confirm statistically significant findings in the Kruskal-Wallis test, the Mann-Whitney U test was applied. P values of <0.05 in the Mann-Whitney U test were defined as statistically significant and encoded in figures (*, P < 0.05; **, P < 0.01). All statistical analyses were performed using SPSS 12.0 software (SPSS).

### Results

**Inhibition of SOCS-3 expression in prostate cancer cells.** To investigate the role of SOCS-3 in regulation of prostate cancer cell death, we used a siRNA approach. To obtain a high-transfection efficiency and maintain low SOCS-3 mRNA and protein levels, we treated SOCS-3–positive DU-145, LNCaP-IL-6+, and PC3 cell lines twice with either 2 nmol/L of specific siRNA against SOCS-3 (25) or control siRNA during 6 days. On day 6, the cells were harvested and SOCS-3 mRNA and protein expression levels were measured by quantitative reverse transcription–PCR (qRT-PCR) and Western blot, respectively. We found a 70% to 80% decrease of SOCS-3 mRNA (Fig. 1A) and protein (Fig. 1B) compared with the controls in all three cell lines.

**Decrease of SOCS-3 leads to reactivation of STAT3 and STAT1.** In previous studies, we showed that STAT3 could not be activated by IL-6 in SOCS-3–positive prostate cancer cell lines (22). To elucidate if SOCS-3 down-regulation is sufficient for STAT3 reactivation, we treated DU-145 and LNCaP-IL-6+ cells with 20 ng/mL IL-6 for 15 min (Fig. 1C). The SOCS-3–negative parental cell line LNCaP ATCC was used as a positive control. In that cell line, phosphorylation of STAT3 can be induced by IL-6. SOCS-3 down-regulation was sufficient to reactivate STAT3 in DU-145 and LNCaP-IL-6+ cells. Because modulation of SOCS-3 expression may affect other members of the STAT family, we examined the implications of siRNA treatment on the levels of phosphorylated STAT1. Interestingly, we found that down-regulation of SOCS-3 enhances STAT1 phosphorylation in LNCaP-IL-6+ and DU-145 cells (Supplementary Fig. S1).

**SOCS-3 down-regulation causes growth inhibition.** To investigate the consequences of SOCS-3 down-regulation, cell proliferation was measured using [3H]thymidine incorporation assay for DU-145, LNCaP-IL-6+, and PC3 cells (Fig. 2). The LNCaP ATCC cell line was used as a negative control (Fig. 2). All three cell lines responded to a down-regulation of SOCS-3 by a significant 50% to 80% decrease in cell proliferation. As expected, there was no change in the proliferation of the LNCaP ATCC cell line. In concordance with a significant decrease in proliferation, we also observed a change in cell morphology and decreased viability of cells treated with specific siRNA, compared with cells treated with control siRNA or with LNCaP ATCC cells, which did not change in morphology or viability at all (Fig. 3A).

**Cells with decreased SOCS-3 levels undergo apoptosis.** Because most of these cells seemed dead under the microscope, we hypothesized that changes in the apoptosis rate occur. Apoptosis was measured with PI staining 3 days after the second transfection. We found that a significant number of DU-145, LNCaP-IL-6+, and PC3 cells treated with specific siRNA against SOCS-3 underwent apoptosis, whereas only a few apoptotic cells were observed in the controls (Fig. 3B). LNCaP ATCC cells did not show an increase of apoptosis after treatment with SOCS-3–specific siRNA. Furthermore, the activities of the key executioners of both apoptotic pathways, caspase-3/caspase-7, caspase-8, and caspase-9, significantly increased in DU-145 and LNCaP-IL-6+ (Fig. 4A) but not in the LNCaP ATCC cell line. In the cell lines, the activity of caspase-3 was four times, the activity of caspase-8 was up to four times, and that of caspase 9 was about twice higher than in the controls. The p85 fragment of PARP, cPARP, which is cleaved by activated caspase-3 and represents the end point of the intrinsic apoptosis pathway, was also significantly increased in cells treated with specific siRNA in all three SOCS-3–positive cell lines, whereas no cPARP expression was detectable in LNCaP ATCC cells (Fig. 4B).

To understand the mechanism, we looked at expression levels of the antiapoptotic proteins Mcl-1 and Bcl-2. In DU-145 and PC3 cells, there were no significant changes in Mcl-1 expression levels, whereas we could observe a significant decrease of the protein in LNCaP-IL-6+ cells (Fig. 5A). Furthermore, Bcl-2 protein levels decreased significantly in DU-145 and PC3 cells to 50% and in LNCaP-IL-6+ cells to ~30% compared with the control siRNA treatment (Fig. 5B).

**Overexpression of Bcl-2 rescues cells with low SOCS-3 levels from apoptosis.** To determine if Bcl-2 is crucial for the survival of cells with low SOCS-3 expression levels, we cotransfected DU-145 and LNCaP-IL-6+ cells with 2 nmol/L control siRNA, siRNA against SOCS-3, or mock and 0.5 μg/mL pEGFP-Bcl-2 or empty vector as indicated twice and measured apoptosis afterwards (Fig. 5C). In both cell lines, we observed a significant decrease in the percentage of apoptotic cells that were transfected with the Bcl-2 vector and siRNA against SOCS-3 compared with cells that were treated with siRNA against SOCS-3 alone.

**SOCS-3 is overexpressed in CRPC.** To get a better insight into SOCS-3 expression levels during cancer development and progression, we implemented TMAs using patient samples (Fig. 6). Only a weak expression of SOCS-3 (29.66% positive cells) was detected in the basal prostate epithelial cell layer. In PIN and carcinoma samples, we could observe an increase of SOCS-3–positive epithelial cells (PIN 58.68%, carcinoma 89.32%; Fig. 6A) compared with the benign prostate tissue. These differences were found to be statistically significant (P < 0.001). In samples from CRPC patients, expression of SOCS-3 was even higher (benign tissue 46.63%, carcinoma 93.80%, P < 0.001; Fig. 6B). To better understand the relationship between SOCS-3 expression and programmed cell death in vivo, we have performed TMAs for Bcl-2. The highest expression of Bcl-2 was detected in PIN samples obtained from cancer patients, followed by benign tissue (Fig. 6C). A small percentage of Bcl-2–positive cells was detected in tumors, whereas no Bcl-2 positivity was detected in specimens from CRPC patients (Fig. 6D).

### Discussion

Deregulation of expression of STAT3 or activation of the JAK-STAT pathway are implicated in many diseases including cancer. Although recent studies have linked STAT3 to advanced tumor growth and metastatic progression of several carcinomas (28–32), the function of STAT3 in prostate cancer seems to be more complex. Differences in STAT3 phosphorylation reported by various laboratories may be in part explained by results from a recent publication of Kreis and associates. The authors showed that STAT3 activity depends on cell density in melanoma models,
Figure 1. A, SOCS-3 mRNA down-regulation after treatment with siRNA. DU-145, LNCaP-IL-6+, and PC3 cells were treated twice with 2 nmol/L siRNA against SOCS-3 during a period of 6 d. The cells were then harvested, and qRT-PCR was performed. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, \( P < 0.05 \); **, \( P < 0.01 \), Mann-Whitney U test).

B, SOCS-3 protein down-regulation after treatment with siRNA. DU-145, LNCaP-IL-6+, and PC3 cells were treated with SOCS-3 siRNA as described above. The cells were then harvested, and Western blots were performed. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, \( P < 0.05 \), Mann-Whitney U test).

C, STAT3 phosphorylation after SOCS-3 protein down-regulation. After two consecutive transfections with SOCS-3 siRNA, DU-145 and LNCaP-IL-6+ cells were treated with 20 ng/mL IL-6 for 15 min.
and these findings may also be relevant to carcinoma of the prostate (33). The transcription factor has been implicated in promotion of apoptosis or growth arrest under certain conditions (4, 34, 35). However, the molecular basis for such a dual function of STAT3 has not been clarified yet. It is known that various regulators of STAT3, such as protein inhibitors of activated STAT (PIAS) and the family of SOCS proteins, can determine the activation status of this transcription factor. In previous studies, we were able to show that STAT3 phosphorylation inversely correlates with SOCS-3 expression in vitro. In prostate cancer cell lines that express SOCS-3, STAT3 could not be phosphorylated (22). In the present manuscript, we investigated biological effects of SOCS-3 down-regulation in DU-145, LNCaP-IL-6+al, and PC3 prostate cancer cell lines, which are considered models for an advanced disease. Down-regulation of SOCS-3 with specific siRNA resulted in restoration of STAT3 phosphorylation induced by IL-6 in DU-145 and LNCaP-IL-6+ cells. Thus, down-regulation of SOCS-3 seems to be crucial for STAT3 phosphorylation in these cell lines. These data are consistent with the in vitro results of Lu and colleagues, who showed STAT3 phosphorylation after down-regulation of SOCS-3 in mouse embryonic fibroblasts. Furthermore, they proved that loss of SOCS-3 gene expression converts the antiapoptotic function of STAT3 into proapoptotic (35). It is known that SOCS-3 is an important feedback regulator of the IL-6/JAK-STAT pathway and SOCS-3 expression can be induced by IL-6, cyclic AMP, and androgens (22, 23). Cell lines in the present study in which apoptosis was induced were IL-6-positive, and therefore, our results support the contention that IL-6-induced apoptosis through STAT3 phosphorylation occurs after SOCS-3 inhibition.

In prostate cells with elevated levels of SOCS-3, we observed a suppression of androgen-regulated cell growth (23). In the present manuscript, we show for the first time that down-regulation of SOCS-3 leads to a decrease in proliferation of prostate cancer cells. Taken together with the results of our previous study (23), one could hypothesize that different functions of SOCS-3 in prostate cancer depend on the presence of the androgen receptor (AR). Decreased SOCS-3 levels result in an increase of apoptosis in all tested SOCS-3-positive prostate cancer cell lines in which AR expression is absent. SOCS-3 expression in AR-positive cell lines is heterogenous; we show in the present paper that VCaP cells are, similar as LNCaP, SOCS-3 negative (Supplementary Fig. S2), whereas LAPC-4 cells express SOCS-3 protein (22). In VCaP cells, induction of pSTAT3 after IL-6 treatment was observed. Other studies have indicated that SOCS-3 effects are not limited solely to IL-6 signaling. Masuhiro and associates showed that SOCS-3 is an important key regulator in cell cycle progression (36). It is influenced by mutual interaction between SOCS-3 and the transcriptional factor DP-1. Therefore, it seems that SOCS-3 is implicated in the regulation of cell cycle and programmed cell death. We also showed that increased apoptosis after SOCS-3 down-regulation is associated with a significant increase of active proapoptotic caspase-8, caspase-9, and caspase-3/caspase-7, key proteases of both apoptosis pathways. Induced caspase activity was correlated with increased levels of cPARP. Cell survival is dependent on a complex interplay between proapoptotic and antiapoptotic proteins. To understand the mechanism responsible for increased apoptosis after SOCS-3 down-regulation, we looked for changes in the expression levels of the antiapoptotic proteins of the Bcl-2 family, Mcl-1, and Bcl-2. In previous studies, it was shown that Mcl-1 expression increases during tumor progression, whereas contradictory results on Bcl-2 are presented in the literature (37–40). In a recent publication, we showed that Mcl-1 is regulated by
Figure 3. SOCS-3 protein down-regulation causes a decrease in cell viability and increase in the apoptotic rate after treatment with specific siRNA in all SOCS-3–positive cell lines but not LNCaP ATCC cells. A, DU-145, LNCaP-IL-6+, PC3, and LNCaP ATCC cells were treated twice with control siRNA or siRNA against SOCS-3. On day 6, the pictures of all cells were captured (magnifications, 40× and 200×). B, determination of the apoptosis rate in DU-145, LNCaP-IL-6+, PC3, and LNCaP ATCC cells. The apoptotic rate was assessed by PI staining and flow cytometry (mean values ± SEM from at least three independent experiments; *P < 0.05; **P < 0.01, Mann-Whitney U test).
IL-6 and mediates survival activity of the cytokine in models of late stage prostate carcinoma (41). In the present study, no alteration in expression levels of Mcl-1 could be observed in DU-145 and PC3 cells after SOCS-3 down-regulation. Only in the LNCaP-IL-6+ cell line, a down-regulation of SOCS-3 was associated with a decrease of Mcl-1. Thus, regulatory mechanisms in prostate cancer differ from those in cholangiocarcinoma cells in which SOCS-3 inhibits IL-6 induction of Mcl-1 (42). In contrast, Bcl-2 levels decreased significantly in all tested prostate cancer cell lines. These findings could be discussed in view of published results of Vanasse and colleagues (43). They showed that overexpression of Bcl-2 results in induction of SOCS-3 in mouse embryonic fibroblasts and hematopoietic cell lines suggesting a Bcl-2-associated mechanism underlying SOCS-3 induction. A colocalization of Bcl-2 and SOCS-3

Figure 4. Increased activation of the proapoptotic caspase-3, caspase-8, and caspase-9 and PARP cleavage after SOCS-3 down-regulation. A, DU-145, LNCaP-IL-6+, and LNCaP ATCC cells were treated with SOCS-3 siRNA. A significant increase of the caspase-3/caspase-7, caspase-8, and caspase-9 activity could be observed in the SOCS-3–positive cell lines LNCaP-IL-6+ and DU-145, but not in the control cell line LNCaP ATCC. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, P < 0.05; **, P < 0.01, Mann-Whitney U test). B, cPARP expression levels after SOCS-3 down-regulation. DU-145, LNCaP-IL-6+, PC3, and LNCaP-ATCC cells were treated with siRNA against SOCS-3. cPARP expression significantly increased in all three SOCS-3–positive cell lines. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, P < 0.05; **, P < 0.01, Mann-Whitney U test).
could be observed in de novo follicular lymphoma but not in benign tissue (43). Thus, it was concluded that induction of SOCS-3 is an early genetic event, influencing pathways important for the pathogenesis of follicular lymphoma. Nevertheless, we did not observe an induction of SOCS-3 after overexpression of Bcl-2 in prostate cancer cell lines (data not shown). To investigate if down-regulation of Bcl-2 is a trigger for increased apoptosis in SOCS-3-deficient cells, we overexpressed Bcl-2 in DU-145 and LNCaP-IL-6+ cells. A decrease in the percentage of apoptotic cells in both cell lines indicates that restored Bcl-2 expression levels could prevent cells lacking SOCS-3 from undergoing apoptosis. In PC3 cells in which Bax expression was shown, the Bcl-2/Bax ratio is therefore altered (44). In contrast, DU-145 and LNCaP-IL-6+ cells express Bcl-2 but not Bax (45). In future studies, it may be determined how SOCS-3 interacts with various proteins in the apoptosis pathways and which apoptosis pathway is initially activated.
In a previous study, we reported on increased SOCS-3 staining intensity in prostate cancer versus benign tissue (22). We could confirm and extend those previous data. In PIN and malignant tissue, the expression of SOCS-3 was up-regulated. The highest expression was found in tissue specimens of CRPC patients. An increase of SOCS-3 in prostate cancer development and progression can be explained by elevated IL-6 levels during carcinogenesis. It is well known that IL-6 exhibits pleiotropic effects in vitro and in vivo and SOCS-3 is the main negative feedback regulator of IL-6–mediated JAK-STAT activation. On the basis of the in vitro and in vivo results of the present study, we hypothesize that SOCS-3 not only has a classic negative feedback regulator function, but it is crucial for cancer cell survival in CRPC. Interestingly, Tu and colleagues did not detect Bcl-2 in metastatic prostate cancer (40). On the basis of increased expression of Bcl-2 and SOCS-3 in PIN, we hypothesize that the inhibition of apoptosis by SOCS-3 as described in the present manuscript may be particularly important during early malignant transformation in the prostate.

Accumulated evidence suggests that proapoptotic or antiapoptotic effects of SOCS-3 are cell type specific. In mammary gland development, SOCS-3 is essential for cell survival. Sutherland and colleagues could prove that silencing of the SOCS-3 gene leads to accelerated apoptosis through activation of STAT3 and induction of proapoptotic Bak and Bax proteins (46, 47). In many tumors, SOCS-3 is silenced through gene promoter hypermethylation. This leads to an increased proliferation and tumor growth (18–20). In those carcinomas, overexpression of SOCS-3 results in suppression of cell growth. Thus, it has been proposed that SOCS-3 has a tumor suppressor function. In contrast to those data, SOCS-3 is constitutively expressed in breast cancer and does not suppress growth of MCF-7 tumor cells (48). In a recent publication, it was however shown that SOCS-3 inhibits proliferation of T47D cells (49).

Taken together, in the present manuscript, we show for the first time that SOCS-3 is important for the survival machinery of prostate cancer cells and its down-regulation leads to increased
cell death through activation of the extrinsic and intrinsic apoptosis pathways. SOCS-3 expression is increased during development and progression of prostate cancer in clinical specimens. On the basis of the present results, we suggest that SOCS-3 may be a potential target for future therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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