Published in final edited form as:

Clin Cancer Res. 2009 December 15; 15(24): 7634–7641. doi:10.1158/1078-0432.CCR-09-0264.

Transforming growth factor- β -stimulated clone-22 (TSC-22) is an androgen regulated gene that enhances apoptosis in prostate cancer following IGF-IR inhibition

Cynthia C. T. Sprenger^{1,2}, Kathleen Haugk³, Shihua Sun¹, Ilsa Coleman⁴, Peter S. Nelson^{1,4}, Robert L. Vessella^{2,3}, Dale L. Ludwig⁵, Jennifer D. Wu¹, and Stephen R. Plymate^{1,3}

¹Department of Medicine, University of Washington, Seattle, Washington

²Department of Urology, University of Washington, Seattle, Washington

³Puget Sound Veterans Affairs Health Care System, Seattle, Washington

⁴Fred Hutchinson Cancer Research Center, Seattle, Washington

⁵Imclone Systems Incorporated, New York, New York

Abstract

Purpose—Inhibition of IGF signaling using the human IGF-IR monoclonal antibody A12 is most effective at inducing apoptosis in prostate cancer xenografts in the presence of androgen. We undertook this study to determine mechanisms for increased apoptosis by A12 in the presence of androgens.

Experimental Methods—The castrate-resistant human xenograft LuCaP 35V was implanted into intact or castrate SCID mice and treated with A12 weekly. After six weeks of tumor growth animals were sacrificed and tumors removed and analyzed for cell cycle distribution/apoptosis and cDNA arrays were performed.

Results—In castrate mice the tumors were delayed in G2 with no apoptosis; in contrast tumors from intact mice underwent apoptosis with either a G1 or G2 delay. TSC-22 was significantly elevated in tumors from the intact mice compared to castrate mice, especially in those tumors with the highest levels of apoptosis. In order to further determine the function of TSC-22, we transfected various human prostate cancer cell lines with a plasmid expressing TSC-22. Cell lines overexpressing TSC-22 demonstrated an increase in apoptosis and a delay in G1. When these cell lines were placed subcutaneously in SCID mice a decreased number of animals formed tumors and the rate of tumor growth was decreased compared to control tumors.

To whom correspondence should be addressed: Stephen R. Plymate, MD, 325 9th Avenue, Box 359625, Seattle WA 98104, splymate@u.washington.edu.

Clinical Relevance: In this study, we show that androgen-regulated genes such as transforming growth factor- β -stimulated clone-22 (TSC-22) may be responsible for the induction of apoptosis following inhibition of the insulin-like growth factor receptor 1 (IGF-IR) in androgen-dependent prostate cancer. The induction of TSC-22 by androgen in castrate-resistant prostate cancer xenografts that are placed in an intact host may reinitiate an apoptotic response to IGF-IR inhibition that is lost when the same xenografts are placed in castrate hosts. Clinically both fully human monoclonal antibodies, such as A12, and small molecules are in trials for prostate as well as other epithelial malignancies. The timing for the best effects of IGF-IR targeted therapy is important in order to achieve optimal tumor regression. In this study we have identified one factor, TSC-22, whose expression in androgen-dependent disease could indicate a favorable response to early IGF-IR inhibition in prostate cancer.

Conclusions—These data indicate that IGF-IR inhibition in the presence of androgen has an enhanced effect on decreasing tumor growth, in part, through increased expression of the tumor suppressor gene TSC-22.

Keywords

castrate-resistant PCa; androgen; IGF-IR; A12; TSC-22

Introduction

We have shown that inhibition of the type 1 insulin-like growth factor receptor (IGF-IR) with the fully human monoclonal antibody A12 resulted in a decreased rate of tumor growth for both androgen-dependent (AD) and –independent (AI) human prostate cancer xenografts (1, 2). However, depending on whether or not the xenograft was castration-resistant, we saw either apoptosis, for AD xenografts grown in intact mice, or a slowing of tumor growth due to G2 arrest without apoptosis, for androgen-independent (AI) xenografts grown in castrate mice. Thus, inhibition of signaling through IGF-IR resulted in significant suppression of prostate tumor growth by both increased apoptosis and decreased proliferation (1). Furthermore, we demonstrated that inhibition of IGF-IR signaling in AD xenografts following castration significantly delayed the time to progression to androgen-independent prostate cancer (3). A further suppression of androgen receptor (AR) transcriptional activity also occurred following A12 treatment (3). In this current study we investigated whether the differences in apoptosis between AD and AI human prostate cancer xenografts were due to the presence of androgen in the AD animals or to a change in tumor phenotype.

We found an increase in transcript levels of several androgen-regulated genes when castrate-resistant LuCaP 35V tumors were grown in intact mice. One androgen-regulated gene of particular interest was transforming growth factor- β_1 -stimulated clone-22 (TSC-22). TSC-22, which is regulated by androgens, TGF β_1 , and PPAR γ , has been associated with decreased tumor formation and thus is a putative tumor-suppressor gene (4-10). In this study we demonstrate that TSC-22 is an androgen regulated tumor-suppressor gene and that inhibition of IGF-IR signaling increased TSC-22 expression in a human xenograft model of prostate cancer. We further show that expression of TSC-22 in three human prostate cancer cell lines increased the apoptotic response, which may account, in part, for our previous observation of an apoptotic response to IGF-IR inhibition in AD prostate tumor xenografts (1).

Materials and Methods

Xenografts and cell lines

The LuCaP 35V AI human prostate xenograft, which only grows *in vivo*, was originally described by Corey *et al* (2003) (11). The generation and characterization of the M12 cell line has been described previously (12-14). The PC-3 cell line was obtained from ATCC (Rockville, MD). Both cell lines were cultured in RPMI1640 medium supplemented with 5% FCS, 10 ng/ml epithelial growth factor (EGF), 0.02 mM dexamethasone, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenium, fungizone, and gentamicin at 37°C with 5% CO₂. The androgen-receptor positive LNCaP C4-2 subline was a gift from Dr. Robert Sikes (University of Delaware, Newark, DE). These cells were grown in T-medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37°C with 5% CO₂.

Cell transfection

A human full-length cDNA clone of TSC-22 (NM_006022) was obtained from Origene Technologies (Rockville, MD) and subcloned into pcDNA 3.1 with a G418 resistance gene. M12, PC-3, and the LNCaP C4-2 human prostate cancer cells were transfected with either control pcDNA 3.1 vector or pcDNA-TSC-22 plasmid. Transfections were performed with Lipofectin 2000™ (Promega, Madison, WI) according to the manufacturer's protocol. Stable clones were obtained with G418 selection (400 µg/ml) and the expression of TSC-22 was confirmed by Western blotting.

RNA interference

Small hairpin RNAs (shRNAs) targeting TSC-22 mRNA were designed and purchased from Origene (Rockville, MD). The shRNA sequence resulting in the most inhibition was shTSC22D1: 5'CCTCATTTGCCTCACCTTCCACAACAGAA3'. shTSC22D1 was transfected into LNCaP C4-2 cells using Lipofectamine (Invitrogen, Carlsbad, CA). Levels of TSC-22 were measured three days post-transfection. For apoptosis studies, transfected cells were treated 72 hrs post-transfection with A12, IGF, and DHT for 6-48 hours.

Animal Studies

The growth of LuCaP 35V in castrate mice and responses to A12 treatment have been previously reported from our laboratory (1). The RNA used for microarray analysis in this study was collected immediately after the tumor was removed and was preserved in DEPC H₂O at -80°C. For studies of LuCaP 35V growth and response to A12 in non-castrate (intact) animals, tumor bits (20 to 30 mm³) of LuCaP 35V were implanted subcutaneously into ten six to eight-week-old intact SCID mice as previously described for the LuCaP 35 xenografts (1). When the implanted tumor reached a volume of 100 mm³, half the animals received A12 antibody intraperitoneally (ip) at a dose of 40 mg/kg body weight three times a week for up to five weeks and the other half received human IgG as a control. Animals were weighed twice a week. Blood samples were collected from orbital sinus weekly. Plasma was separated and PSA level was determined using the IMx Total PSA Assay (Abbott Laboratories, Abbott Park, IL). Tumors were measured twice weekly and tumor volume was estimated by the formula: volume = (1 × w²)/2. After euthanization, tumors were collected, quartered, and treated as follows: 1) fixed in 10% neutral buffer formalin (NFB), embedded in paraffin, and five micron sections prepared for immunohistochemistry (IHC); 2) separated into single cells mechanically by mincing and filtering through 70µm nylon sieves for flow cytometry; 3) minced and extracted for protein blots; and 4) RNA extracted and cDNA prepared for microarrays as previously described (1,15). For TSC-22 studies, groups of ten male athymic, nude mice were subcutaneously injected with a million cells each of either empty vector control cells (M12pc) or TSC-22 overexpressing cells (M12 TSC-22). Mice were monitored weekly for tumor formation; tumors were measured and tumor volume was estimated as described above. Animals were euthanized after seven weeks and tumors removed. All animal studies performed followed a University of Washington approved IACUC animal protocol.

cDNA microarray analyses

Custom PEDB cDNA microarrays were constructed as previously described using clones derived from the Prostate Expression Data Base (16), a sequence repository of human prostate expressed sequence tag (EST) data available to the public (www.pedb.org). The inserts of individual cDNA clones were amplified by polymerase chain reaction (PCR), purified, and spotted in duplicate onto glass microscope slides (Gold Seal, Becton-Dickinson) with a robotic spotting tool (GeneMachine OmniGrid 100). Labeling with Cy3 and Cy5 fluorescent dyes and hybridization to the microarray slides were essentially as described (17). Fluorescent array images were collected for both Cy3 and Cy5 using a GenePix 4000B fluorescent scanner

(Molecular Devices, Sunnyvale, CA). The image intensity data were gridded and extracted using GenePix PRO 4.1 software, and spots of poor quality determined by visual inspection were removed from further analysis. RNA from tumors from A12-treated castrated mice was pooled into two groups: 1) tumors that exhibited G2-arrest (8 tumors) and 2) tumors that exhibited no G2 arrest (10 tumors). These two pools were hybridized against a pool of 18 tumors from untreated castrated tumor controls. RNA from tumors from A12-treated intact mice was pooled into three groups: 1) G1 arrest with apoptosis (2 tumors), 2) G2 arrest with apoptosis (2 tumors), and 3) G1 arrest with no apoptosis (2 tumors). These three pools were hybridized against a pool of four untreated non-castrated tumor controls. RNA also was collected from laser-captured microdissections from 37 human prostate cancer tissue samples. Each sample yielded RNA from two areas: benign and cancer. Each of these experiments (LuCaP 35V RNA and human PCa RNA) was repeated with a switch in fluorescent labels to account for dye effects. Normalization of the Cy3 and Cy5 fluorescent signal on each array was performed using GeneSpring 7.3 software (Agilent Technologies, Santa Clara, CA.) A print-tip specific Lowess curve was fit to the log-intensity versus log-ratio plot and 20.0% of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than 10, then 10 was used instead. Data were filtered to remove values from poorly hybridized cDNAs with average foreground minus background intensity levels less than 300. Data from the two duplicate cDNAs spots on each PEDB chip were combined and the average ratios were used for comparative analyses. Ratios were filtered to include only clones whose expression was measurable in at least 75% of the samples. Differences in gene expression were determined using the SAM procedure (18), (<http://www-stat.stanford.edu/~tibs/SAM/>). Gene expression differences with a false discovery rate (FDR) of $\leq 1\%$ were considered significant.

Immunohistochemistry

Tumor samples were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned at 5 μm onto slides. After deparaffinization and rehydration, antigens were retrieved with 0.01 mol/L citric acid (pH 6.0) at 95°C for 2 to 5 minutes. Slides were allowed to cool for 30 minutes, followed by sequential rinsing with PBS. Endogenous peroxidase activity was quenched by an incubation with 0.3% H_2O_2 in methanol for 15 minutes. After blocking with 1.5% normal goat serum in PBS containing 0.05% Tween 20 (PBST) for 1 hour, slides were incubated with TSC-22 antibody (1 $\mu\text{g}/\text{mL}$) for 1 hour followed by sequential incubation with biotinylated goat anti-mouse IgG for 30 minutes, peroxidase-labeled avidin for 30 minutes (Santa Cruz Biotechnology), and diaminobenzidine/hydrogen peroxide chromogen substrate (Vector Laboratories, Burlingame, CA) for 5 to 10 minutes. Negative controls were performed with TSC-22 antibody pre-absorbed with TSC-22 protein (Abnova, Taipei, Taiwan). All incubation steps were done at room temperature. Slides were counterstained with hematoxylin (Sigma, St Louis, MO), and mounted with permount (Fisher Scientific, Fair Lawn, NJ).

Western blotting

For cells in culture, cells were washed with PBS and lysed with cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, 10% Triton X-100) containing Phosphatase Inhibitor Cocktail II (Sigma) and protease inhibitors (Complete Mini Tablets™, Roche, Indianapolis, IN). For assaying *in vivo* effects of A12, freshly prepared xenografts were minced then washed with PBS and lysed as described for cell culture. Twenty-five micrograms of protein was resolved on 4-15% SDS-PAGE gels, transferred onto a nitrocellulose membranes, and probed with a 1:400 dilution of a rabbit polyclonal TSC-22 antibody (ProteinTech Group, Chicago, IL). The blot was washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Pharmacia Biotech, Piscataway, NJ) for 1 h. Immunoreactive proteins were detected by ECL (Pharmacia Biotech). The membranes were stripped for 30 minutes in stripping buffer (Pierce, Rockford, IL) and re-probed with anti- β

actin antibody (Chemicon, Temecula, CA) as described above. Independent experiments validated that this stripping procedure did not lead to loss of signal.

Apoptosis and cell cycle assay

Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay using the Apop-Direct kit (BD BioScience) as previously described (19). Briefly, 1×10^6 cells from the single-cell suspension were fixed with 10% neutral buffer formalin (NBF) followed by 70% ethanol alcohol at -20°C for 30 min. After several washes, cells were permeabilized with 0.1% Triton X-100 and incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme (TdT) at 37°C for 1 h, followed by an incubation with a propidium iodide/RNase buffer (100 $\mu\text{g}/\text{ml}$ of propidium iodide (PI), 50 $\mu\text{g}/\text{ml}$ RNase) at room temperature for 60 min. Cell cycle was determined by staining separate cell aliquots with propidium iodide as previously described (1). Samples were analyzed by flow cytometry using a BD FACscan (BD BioScience, San Jose, CA). Data were analyzed with CellQuest^{PRO} software. Apoptosis was also measured via Western blots using antibodies against the intact and cleaved forms of PARP and caspase-3 and -7 (Cell Signaling Technologies, Danvers, MA). The presence of cleavage products with a concomitant decrease in levels of intact protein for any of these three proteins indicates apoptosis has occurred.

Cell proliferation assay

Cells were seeded in a 96-well plate at 2,500 cells/well in RPMI T&S media for 24 h before addition of 1 or 5 ng/ml TGF- β , 10^{-8} DHT, 20 ng/ml IGF-I, 40 $\mu\text{g}/\text{ml}$ A12, DHT + IGF, DHT + A12, IGF + A12, or DHT + IGF + A12. When A12 was used, it was added one hour prior to addition of other factors. Proliferation was quantified after 72 hours by a colorimetric MTS tetrazolium (MTT) assay using the Cell Titer 96 AQueous kit (Promega) according to the manufacture's protocol. Eight replicates were performed in each experiment and each experiment was performed three times.

Real-Time PCR

M12, PC3, and LNCaP C4-2 cells were treated with IGF-I (1, 10, 50 ng/ml), DHT, and A12 (40 $\mu\text{g}/\text{ml}$) for 3 hrs. Then RNA was collected and real-time PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PR System using ABI SYBR Green 2 $\times$ MasterMix (ABI, Foster City, CA). Primers used:

IGF-IR Forward (5'GAAGTGGAACCCTCCCTCTC3')

IGF-IR Reverse (5'CTTCTCGGCTTCAGTTTGG3')

TSC-22 Forward (5'GAAATGTTGTCCACAAGAGTGTC3')

TSC-22 Reverse (5'TGCTGAGGAGACATTCGGCTG3')

Results

Growth and PSA levels of LuCaP 35V tumors in castrate and non-castrate mice

A12 treatment significantly reduced tumor volume and PSA levels of LuCaP 35V tumors grown in non-castrate animals, $p < 0.01$ (Figure 1A, B). Further, LuCaP 35V tumor volumes were significantly lower in the A12 treated intact mice than in the A12 treated castrate mice, $p < 0.05$ (Figure 1A). This decrease in tumor volume was accompanied by a significant decrease in serum PSA in the treated intact mice compared to the treated castrate mice, $p < 0.05$ (Figure 1B).

Cell cycle and apoptosis in xenografts

In contrast to the studies in castrate animals in which LuCaP 35V animals treated with A12 had smaller tumors due to an arrest in G2, 60 percent of the LuCaP 35V tumors treated with A12 in intact animals underwent both apoptosis and cell cycle arrest, either G1 or G2 (Figure 1C-F).

The transcriptional program of LuCaP 35V tumors treated with A12

To identify factors that may be responsible for apoptosis, we examined those androgen regulated genes that were differentially regulated in tumors undergoing apoptosis versus non-apoptotic tumors from either intact or castrate mice and their regulation in each set of animals by A12 (Figure 2A). In the intact group that underwent apoptosis, A12 elicited a significant increase in a number of androgen-regulated genes, including TSC-22, which is of particular interest because it has been associated with the induction of apoptosis and it is a potential tumor-suppressor gene for salivary, glial, and prostate cancers (4-7, 9, 20, 21). As would be expected for an androgen induced gene, TSC-22 mRNA levels were significantly higher in xenografts grown in intact mice (both treated and untreated) compared to xenografts grown in treated castrate mice ($p < 0.01$); mean mRNA levels of TSC-22 in xenografts from treated intact mice were also higher than those from the untreated intact mice, $p < 0.05$ (Figure 2B). Immunohistochemistry on tumors demonstrated a decrease in TSC-22 levels in the castrate hosts compared to intact hosts (Figure 2C). Using Western blot, we then demonstrated an increase in TSC-22 protein levels for tumors that underwent apoptosis following A12 treatment (Figure 2D).

TSC-22 expression in benign and malignant human prostate glands

In order to determine the clinical relevance of TSC-22 in prostate cancer, we examined laser capture dissected prostate RNA from 37 patients with prostate cancer (Gleason 6-10) (22). Benign and malignant glands were dissected from each individual so each gland served as its own control. Amplified cDNA made from the RNA was then examined by cDNA microarray analysis as described in Materials and Methods. Analysis of TSC-22 demonstrated a significant decrease in mRNA expression compared to normal tissue in 30 of 37 glands, $p < 0.0001$ (Figure 3). This finding is consistent with a recent report using immunohistochemistry demonstrating that TSC-22 protein was also significantly decreased in malignant versus benign prostate epithelium (10).

In vitro effects of TSC-22 in the M12, LNCaP C4-2, and PC-3 human prostate cancer cell lines

Since TSC-22 is an androgen regulated and potentially an IGF-IR negatively regulated gene, we treated the androgen receptor positive LNCaP C4-2 cell line with both DHT and IGF-I and then exposed the cells to either A12 or IgG. DHT exposure significantly increases levels of IGF-IR in AR positive prostate cancer cells, including the LNCaP cell lines, but has no effect in AR negative lines such as PC3 (23,24). In LNCaP C4-2 cells grown in the absence of androgen TSC-22 is detectable but levels are markedly lower than in cells grown in the presence of androgen. Real-time PCR demonstrated a significant decrease in the mRNA levels of TSC-22 following IGF-I treatment and this decrease was blocked by the addition of A12 (Figure 4A). Proliferation assays on these cells following treatment with DHT and A12 demonstrated that A12 blocked the DHT and DHT + IGF-1 induced increase in proliferation (Figure 4B). Finally, when we used shRNAs to knock-down TSC-22 levels in the LNCaP C4-2 cells we saw decreased apoptosis following DHT + A12 treatment (Figure 4C). Thus the *in vitro* data with the AR positive LNCaP C4-2 line reflected what we observed with the LuCaP 35V xenografts *in vivo*. For the two lines that do not express an androgen receptor, M12 and PC3, no TSC-22 to very little was detected with PCR or Western blot and levels were not

altered by exposure to DHT, IGF-I, or A12 (data not shown); since both of these lines are AR negative and are poorly responsive to exogenous IGF-I, these results were expected.

Because TSC-22 expression has been associated with increased apoptosis in other cancers, we overexpressed TSC-22 in the M12, LNCaP C4-2, and PC-3 cell lines (Figure 5A) and then evaluated the effect of increased expression of TSC-22 on response to A12 and TGF- β treatment. Elevated expression of TSC-22 increased apoptosis in response to A12 and TGF- β treatment as shown by the significantly increased amount of cleaved caspase-3 and -7 and PARP and decreased levels of intact protein ($p \leq 0.01$; Figure 5B) and by flow cytometry (Figure 5C). Further all of the cell lines overexpressing TSC-22 had significantly decreased cell proliferation, $p < 0.05$ (Figure 5D).

***In vivo* tumorigenicity of the M12 TSC-22 cells**

To assess the effect of TSC-22 expression on tumorigenicity of prostate cancer cells, we injected male athymic, nude mice subcutaneously with either 1×10^6 empty vector control M12 cells (M12pc) or the stably transfected M12 TSC-22 cells. Seven weeks post-injection, four out of ten animals (40%) injected with M12 TSC-22 cells had developed tumors compared to eight of ten animals injected with M12pc cells (80%). Further the tumors in the TSC-22 injected animals were significantly smaller than those in the animals injected with the control cells, $p < 0.001$ (Figure 6).

Discussion

Therapy directed towards inhibition of IGF-IR is currently in clinical trials for a number of malignancies. Preclinical data suggest that it may be a successful adjunctive therapy for solid tumors, including prostate cancer (1,2,3). In preclinical prostate xenograft studies, we have shown that when the fully human monoclonal IGF-IR antibody A12 is used as a single agent therapy, the response to IGF-IR inhibition differs depending on whether the tumor is androgen-dependent (AD) (grown in an intact host) or androgen-independent (AI) (grown in a castrate host) (1,3). In this study, in order to determine if it was the innate change in the tumor as it proceeded from AD to AI or the decrease in androgens resulting from castration that accounted for the difference in response to A12, we grew the castrate-resistant LuCaP 35V xenograft in an intact host and demonstrated that the xenograft underwent apoptosis when exposed to A12. These data suggest that genes regulated by the AR-driven transcription program, in some fashion, sensitize the cells to undergo apoptosis after inhibition of the IGF-IR.

The first of these potential genes is the IGF-IR itself. Others and we have shown that the IGF-IR is increased in prostate cancer cells by activation of the androgen receptor (1,23,24). However, there is no evidence that the level of androgen receptor in and of itself determines whether or not a cell will undergo apoptosis versus cell cycle arrest. Rather these data suggest that the presence of higher levels of androgen in the intact mice make the cells more sensitive to IGF-IR inhibition and thus to apoptosis. Based on the results of the microarray data several candidate genes that are known to be associated with tumor suppression, including TSC-22, were upregulated in the tumors undergoing apoptosis (8). Thus TSC-22 is likely part of a group of factors responsible for the effects described in this paper.

TSC-22 has been associated with cellular differentiation, shown to be associated with apoptosis in head and neck epithelial cancers, and to act as a transcription repressor in hepatocellular and lymphoid tumors (5,20,25-27). TSC-22 was first identified in prostate cancer as a gene that was upregulated in response to thiazolidinediones in primary prostate cancer cultures (9). Rentsch *et al* (2006) then demonstrated a decrease in TSC-22 protein expression in human prostate cancer (10). In this study, we have shown a decrease in TSC-22 transcripts in laser-capture microdissected prostate cancer compared to normal epithelium from the same gland.

Nelson *et al* (2002) first demonstrated that TSC-22 was a gene that was significantly increased by androgens in the prostate epithelium (8). In this study, we demonstrate that TSC-22 is increased in the LuCaP 35V xenografts by androgen and further increased by the addition of the human monoclonal IGF-1R antibody A12. Further, we present *in vitro* data demonstrating that TSC-22 is negatively regulated by IGF-I at the mRNA level and that addition of A12 blocks this decrease. *In vivo* the increase in TSC-22 was greatest in those xenografts where apoptosis was clearly evident, suggesting that TSC-22 can induce an apoptotic response in prostate cancer similar to that seen in other tumors. We confirmed this finding *in vitro* by demonstrating a decrease in apoptosis in LNCaP cells expressing a TSC-22 shRNA and an increase in apoptosis following A12 treatment in three human prostate cancer cell lines transfected with TSC-22 expression plasmids. TSC-22 expression also decreased *in vitro* proliferation of these prostate cancer cell lines and decreased *in vivo* tumorigenicity of the M12 TSC-22 cells compared to the M12pc control cells. These results support the role of TSC-22 as an androgen and potentially IGF-1R regulated tumor suppressor in prostate.

This study demonstrates that an increase in TSC-22 expression is part of the mechanism by which IGF-1R inhibition in prostate cancer xenografts induces apoptosis. It further indicates that inhibition of IGF-1R as a therapeutic strategy may be more effective in the presence of androgen or in androgen-dependent disease rather than in the castrate patient with androgen-independent disease.

Acknowledgments

Grants: Supported by NIH Grants CA085859 and CA97186, DOD W81XWH-04-1-0912, and Veterans Affairs Merit Award to SRP and NIH Institutional Training Grant T32 DK07779-08 to CTS

References

1. Wu JD, Odman A, Higgins LM, et al. In vivo effects of the human type I insulin-like growth factor receptor antibody A12 on androgen-dependent and androgen-independent xenograft human prostate tumors. *Clin Cancer Res* 2005;11:3065–74. [PubMed: 15837762]
2. Rowinsky EK, Youssoufian H, Tonra JR, Solomon P, Burtrum D, Ludwig DL. IMC-A12, a human IgG1 monoclonal antibody to the insulin-like growth factor I receptor. *Clin Cancer Res* 2007;13:5549s–55s. [PubMed: 17875788]
3. Plymate SR, Haugk K, Coleman I, et al. An antibody targeting the type I insulin-like growth factor receptor enhances the castration-induced response in androgen-dependent prostate cancer. *Clin Cancer Res* 2007;13:6429–39. [PubMed: 17975155]
4. Choi SJ, Moon JH, Ahn YW, Ahn JH, Kim DU, Han TH. Tsc-22 enhances TGF-beta signaling by associating with Smad4 and induces erythroid cell differentiation. *Mol Cell Biochem* 2005;271:23–8. [PubMed: 15881652]
5. Shostak KO, Dmitrenko VV, Garifulin OM, et al. Downregulation of putative tumor suppressor gene TSC-22 in human brain tumors. *J Surg Oncol* 2003;82:57–64. [PubMed: 12501169]
6. Gupta RA, Sarraf P, Brockman JA, et al. Peroxisome proliferator-activated receptor gamma and transforming growth factor-beta pathways inhibit intestinal epithelial cell growth by regulating levels of TSC-22. *J Biol Chem* 2003;278:7431–8. [PubMed: 12468551]
7. Nakashiro K, Kawamata H, Hino S, et al. Down-regulation of TSC-22 (transforming growth factor beta-stimulated clone 22) markedly enhances the growth of a human salivary gland cancer cell line in vitro and in vivo. *Cancer Res* 1998;58:549–55. [PubMed: 9458104]
8. Nelson P, Clegg N, Arnold H, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci U S A* 2002;99:11890–5. [PubMed: 12185249]
9. Xu Y, Iyengar S, Roberts R, Shappell S, Peehl D. Primary culture model of peroxisome proliferator-activated receptor gamma activity in prostate cancer cells. *J Cell Physiol* 2003;196:131–43. [PubMed: 12767049]

10. Rentsch C, Cecchini M, Schwaninger R, et al. Differential expression of TGF beta-stimulated clone 22 in normal prostate and prostate cancer. *Int J Cancer* 2006;118:899–906. [PubMed: 16106424]
11. Corey E, Quinn J, Buhler K, et al. LuCaP 35: A New Model of Prostate Cancer Progression to Androgen Independence. *Prostate* 2003;55:239–46. [PubMed: 12712403]
12. Bae V, Jackson-Cook C, Maygarden S, Chen J, Plymate S, Ware J. Metastatic sublines of an SV40 large T antigen immortalized human prostate epithelial cell line. *Prostate* 1998;34:275–82. [PubMed: 9496902]
13. Plymate S, Tennant M, Birnbaum R, Thrasher J, Chatta G, Ware J. The effect of IGF system in human prostate epithelial cells of immortalization and transformation by simian virus-40 T antigen. *J of Clin Endocrinol and Metabol* 1996;81:3709–16.
14. York T, Plymate S, Nelson P, Eaves L, Webb H, Ware J. cDNA Microarray analysis identifies genes induced in common by peptide growth factors and androgen in human prostate epithelial cells. *Mol Carcinogenesis* 2005;44:242–51.
15. Plymate S, Haugk K, Sprenger C, et al. Increased manganese superoxide dismutase (SOD-2) is part of the mechanism for prostate tumor suppression by mac25/insulin-like growth factor binding-protein-related protein-1. *Oncogene* 2003;22:1024–34. [PubMed: 12592389]
16. Nelson P, Pritchard C, Abbott D, Clegg N. The human (PEDB) and mouse (mPEDB) prostate expression databases. *Nucleic Acids Res* 2002;30:218–220. [PubMed: 11752298]
17. Bonham M, Arnold H, Montgomery B, Nelson PS. Molecular effects of the herbal compound PC-SPES: identification of activity pathways in prostate carcinoma. *Cancer Res* 2002;62:3920–4. [PubMed: 12124319]
18. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001;98:5116–21. [PubMed: 11309499]
19. Oh Y, Muller H, Ng L, et al. Transforming growth factor-beta-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. *J Biol Chem* 1995;270:13589–92. [PubMed: 7539790]
20. Kawamata H, Fujimori T, Imai Y. TSC-22 (TGF-beta stimulated clone-22): a novel molecular target for differentiation-inducing therapy in salivary gland cancer. *Curr Cancer Drug Targets* 2004;4:521–9. [PubMed: 15379637]
21. Uchida D, Kawamata H, Omotehara F, et al. Over-expression of TSC-22 (TGF-beta stimulated clone-22) markedly enhances 5-fluorouracil-induced apoptosis in a human salivary gland cancer cell line. *Lab Invest* 2000 Jun;80:955–63. [PubMed: 10879745]
22. True L, Coleman I, Hawley S, et al. A molecular correlate to the Gleason grading system for prostate adenocarcinoma. *Proc Natl Acad Sci U S A* 2006;103:10991–6. [PubMed: 16829574]
23. Pandini G, Mineo R, Frasca F, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res* 2005;65:1849–57. [PubMed: 15753383]
24. Plymate S, Tennant M, Culp S, et al. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. *Prostate* 2004;61:276–90. [PubMed: 15368471]
25. Lu Y, Kitaura J, Oki T, et al. Identification of TSC-22 as a potential tumor suppressor that is upregulated by Flt3-D835V but not Flt3-ITD. *Leukemia* 2007;21:2246–57. [PubMed: 17690703]
26. Iida M, Anna CH, Gaskin ND, Walker NJ, Devereux TR. The putative tumor suppressor TSC-22 is downregulated early in chemically induced hepatocarcinogenesis and may be a suppressor of Gadd45b. *Toxicol Sci* 2007;99:43–50. [PubMed: 17533171]
27. Hino S, Kawamata H, Uchida D, et al. Nuclear translocation of TSC-22 (TGF-beta-stimulated clone-22) concomitant with apoptosis: TSC-22 as a putative transcriptional regulator. *Biochem Biophys Res Commun* 2000;278:659–64. [PubMed: 11095965]

Figure 1.

(A) Tumor volume of LuCaP 35V tumors grown in castrate mice compared to growth in non-castrate mice. In both groups, half of the animals were treated with A12, 40 mg/kg per body weight, once the tumor reached 100mm³. Changes in tumor volume after first injection are shown. Note the significant decrease in growth rate of LuCaP 35V tumors grown in non-castrate animals treated with A12 compared to castrate mice (both treated and untreated). (B) Serum PSA levels from the various animal groups. * = $p < 0.01$ for each A12 treated group versus appropriate non-treated group, # = $p < 0.05$ intact + A12 versus castrate + A12. (C-D) Examples of flow cytometry measuring apoptosis of A12 treated LuCaP 35V tumors from a castrate host (C) and an intact host (D). Apoptosis is shown as events above and to the left of the horizontal line. (E-F) Flow cytometry demonstrating the G2 (E) and G1 (F) delay of the A12 treated LuCaP 35V tumors grown in non-castrate animals.

Androgen Regulate Genes

NAME	Castrate				Intact							
	apoptosis →				-				+			
	cycle arrest →				G2				G1			
IGF-IR mAb →	A12	A12	A12	A12	A12	A12	A12	A12	A12	A12	A12	A12
SELENIUM BINDING PROTEIN 1 (SELENBP1) MRNA	0.50	0.60	1.49	1.76	1.60	1.63	0.65	0.72	0.54	0.38		
N-MYC DOWNSTREAM REGULATED GENE 1			-0.27	-0.40	0.82	0.69	0.87	1.16	1.32	1.28		
ENOLASE 1 (ALPHA)	-0.37		-0.22		1.14	1.32	1.39	1.65	1.36	1.23	*	
RAB4A MEMBER RAS ONCOGENE FAMILY	-0.21				1.12	1.16	1.32	1.47	1.16	1.27		
ANKYLOSIS PROGRESSIVE HOMOLOG (MOUSE) (ANKH)		-0.28			0.98	1.24	1.16	1.69	1.37	1.72		
KERATIN 8	-0.66	-0.44	-0.44	-0.29	1.86	1.77	1.36	1.16	1.46	1.44		
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A1 (HNRPA1)	-0.49	-0.56	-0.57	-0.85	0.93	0.94	1.54	1.76	1.42	1.58	*	
TRANSMEMBRANE PROTEASE SERINE 2 (TMPRSS2)		-0.34	-0.79	-0.98	0.87	0.63	1.25	1.51	1.16	1.87	*	
V-MYC MYELOCYTOMATOSIS VIRAL ONCOGENE HOMOLOG (AVIAN)	-0.46	-0.60	-0.58	-0.62	1.91	1.43	2.17	1.85	1.80	1.91		
V-MYC MYELOCYTOMATOSIS VIRAL ONCOGENE HOMOLOG (AVIAN)	-0.61	-0.63	-0.79	-0.80	1.54	2.01	2.09	2.67	2.15	2.19		
SELENIUM BINDING PROTEIN 1 (SELENBP1)	0.21	0.37	0.99	0.99	1.11	1.04	0.65	0.36	0.24	0.22		
UNC-13-LIKE (C. ELEGANS)	0.39	0.39	1.15	1.23	0.83	0.38	0.44	0.40	0.74	0.59		
P53 REGULATED PA26 NUCLEAR PROTEIN	0.29		0.41		1.10	1.23	0.45	0.79	0.63	0.68		
TRANSMEMBRANE PROTEASE SERINE 2 (TMPRSS2)/ PP9284	-0.24	-0.23	-0.63	-0.68	0.49	0.61	1.14	1.34	1.15	1.40	*	
SORBITOL DEHYDROGENASE	-1.37	-0.60	-1.69	-1.13	0.76	0.50	1.66	1.53	1.29	1.09	*	
GLUTAMATE DEHYDROGENASE 1	-1.05	-1.14	-1.28	-1.56	0.42	0.59	1.44	1.86	1.04	0.98	*	
ALDO-KETO REDUCTASE FAMILY 1 MEMBER C2 (DIHYDRODIOL DEHYDROGENASE 2; BILE ACID		0.23	1.03	0.72	1.16	1.82	0.39	NA	0.20	NA		
TRANSFORMING GROWTH FACTOR BETA-STIMULATED PROTEIN TSC-22	-0.27	-0.52	-0.43	-0.53	0.56	0.56	1.17	1.51	1.27	1.77	*	
HUMAN INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 5 (IGFBP5) MRNA	1.56	1.24	1.02	1.05		NA			-0.44	-0.34		
TRANSFORMING GROWTH FACTOR BETA-STIMULATED PROTEIN (TSC22)		-0.32	-0.20	-0.22	0.38	0.50	1.20	1.25	1.15	1.44	*	
TUMOR REJECTION ANTIGEN (GP96) 1	-0.26	-0.27	-0.38	-0.67	0.33	0.51	1.19	1.45	0.65	1.00	*	
N-MYC DOWNSTREAM REGULATED GENE 1	-0.37	-0.27	-0.56	-0.72	0.49	0.48	1.15	1.03	1.13	0.98	*	
NK3 TRANSCRIPTION FACTOR HOMOLOG A (DROSOPHILA)	-0.45	-0.78	-1.24	-1.52			1.11	1.21	0.60	1.22	*	
TUMOR PROTEIN D52	-0.57	-0.69	-0.65	-1.13			1.21	1.53	0.99	1.25	*	
FATTY-ACID-COENZYME A LIGASE LONG-CHAIN 3	-0.48	-0.72	-0.96	-1.21	-0.48	-0.42	0.95	1.30	1.55	1.47	*	
CYCLIN-DEPENDENT KINASE 2	-0.70	-1.16	-0.59	-0.53	0.30	NA	1.16	1.36	1.12	0.69	*	
VAMP (VESICLE-ASSOCIATED MEMBRANE PROTEIN)-ASSOCIATED PROTEIN A 33KDA	-0.58	-0.66	-1.00	-1.36	0.30	0.50	1.50	1.77	0.91	1.24	*	
ATP CITRATE LYASE	-1.00	-0.84	-1.23	-1.40	NA	0.43	1.10	1.89	1.16	0.96	*	
NK3 TRANSCRIPTION FACTOR HOMOLOG A (DROSOPHILA)	-0.66	-0.90	-1.73	-1.73			1.69	1.46	1.17	1.39	*

Figure 2.

(A) cDNA microarray expression values of androgen-regulated genes differentially expressed in apoptotic LuCaP 35V tumors relative to non-apoptotic tumors from both intact and castrate animals. There were 28 genes that changed in the same direction in each pool that were significantly different from each non-apoptotic pool as well as had an FDR of <1%. Identical gene listings (such as TSC-22) indicate different areas of the sequence were spotted on the array. As expected most androgen-regulated genes were increased in the intact versus castrate tumors however an additional set were increased in those tumors treated with A12 in which apoptosis occurred. * $p < 0.05$ apoptosis versus no apoptosis. (B) Results of microarray data for TSC-22 including LuCaP 35V xenografts grown in intact mice. TSC-22 is increased in the xenografts grown in intact mice when compared to castrate mice and TSC-22 expression is increased even further when A12 is added, $p < 0.05$. (C) Immunohistochemistry for TSC-22 protein in representative LuCaP 35V xenografts (1) Negative control with TSC-22 antibody pre-absorbed with TSC-22 protein, (2) untreated intact mouse, (3) A12 treated castrate mouse, and (4) A12 treated intact mouse. Note the increase in staining for TSC-22 in tumors from intact mice (2,4) compared to castrate mice (3) (40× magnification). A representative higher magnification image (200×) is shown for the tumors from intact mice demonstrating positive cytoplasmic staining for TSC-22 compared to castrate host tumors, which have weak staining. (D) Western blot of levels of TSC-22 in apoptotic LuCaP 35V tumors versus non-apoptotic LuCaP 35V tumors from intact mice. β -actin was used as a loading control.

Figure 3.

TSC-22 expression in malignant human prostate glands. Matched benign and malignant glands were laser-capture microdissected from 37 prostate cancer patients and analyzed by cDNA microarray analysis for TSC-22 expression. TSC-22 was significantly down-regulated in 30 out of 37 glands, $p < 0.0001$.

Figure 4.

(A) Quantitative rt-PCR of TSC-22 in LNCaP C4-2 cells treated with IGF-I and dihydrotestosterone (DHT) and A12. Note that IGF-I decreases TSC-22 mRNA levels, * $p < 0.05$ vs 0 or 1 ng IGF-I, and the addition of A12 inhibits this decrease, # $p < 0.01$ vs non-A12 treated cells (+/-SEM). (B) Proliferation assay on LNCaP cells treated as indicated in medium containing 5% CSS. Note the marked enhancement of proliferation with IGF-I and DHT consistent with DHT increasing IGF-IR expression. Effects of DHT and IGF-I are blocked by A12. * = $p < 0.05$ compared to control and # = $p < 0.05$ for IGF+DHT compared to DHT or IGF alone. (C) Knockdown of TSC-22 with RNAi inhibits A12 induced apoptosis. Significant PARP cleavage occurred in the control (scrambled RNAi) but occurred minimally in the TSC-22 RNAi transfected cells following A12 treatment.

Figure 5.

(A) Western blot of TSC-22 expression in M12, LNCaP C4-2, and PC-3 cells transfected with TSC-22 compared to empty vector controls. Clones of the indicated cell lines expressing TSC-22 are shown. β -actin is the loading control. (B) Representative Western blots of caspase-3, -7, and PARP demonstrating increased cleavage of all three proteins over a 48 hr period of culture treatment with TGF- β . Note that an increase in cleavage of these proteins with a concomitant decrease in intact protein is associated with apoptosis. (C) Flow cytometry demonstrating a sub-G₀ peak, indicating apoptosis, in the cells overexpressing TSC-22 (-/+ A12). (D) MTT assay demonstrating the decreased proliferation of cells over-expressing TSC-22 compared to control cells as well as in response to TGF- β . * = $p < 0.01$ versus M12pc control, # = $p < 0.05$ versus M12 TSC-22 control.

Figure 6.

Comparison of average tumor volume seven weeks after subcutaneous injection of either M12pc control cells or M12 TSC-22 cells into male athymic nude mice, $p < 0.001$.