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Expression of TGF β 3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT signaling pathway

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Abstract

Transforming growth factor- β (TGF β) is a secreted cytokine implicated as a factor in cancer cell migration and invasion. Previous studies have indicated that TGF β isoforms may exert differential effects on cancer cells during different stages of the disease, however very little is known about the expression patterns and activity of the three isoforms in prostate cancer. Non-traditional signaling pathways including the PI3-Kinase have been associated with TGF β -mediated effects on cancer cell invasion. In the present study, we have carried out expression analysis of TGF β isoforms and signaling components in cell line models representing different stages of prostate cancer and studied the differential effects of specific isoforms on migratory and invasive behavior and induction of the PI3-kinase pathway. TGF β 1 and TGF β 3 were expressed in all cell lines, with TGF β 3 expression increasing in metastatic cell lines. Both TGF β 1 and TGF β 3 induced motility and invasive behavior in PC3 cells, however, TGF β 3 was significantly more potent than TGF β 1. TGF β RI and Smad3 inhibitors blocked TGF β 1 and TGF β 3 induced motility and invasion. TGF β 3 caused a significant increase in pAKT^{ser473} in PC3 cells and PI3-kinase inhibitor LY294002 blocked TGF β 3 induced migration, invasion and phosphorylation of AKT. Both TGF β RI and Smad3 inhibitors blocked TGF β 3 induced pAKT^{ser473}. Based on these results, we conclude that TGF β 3 is expressed in metastatic prostate cancer cell lines and is involved in induction of invasive behavior in these cells. Furthermore, these effects of TGF β 3 are TGF β RI and Smad3 dependent and mediated via the PI3-kinase pathway.

Keywords

Invasion; Migration; PI3-kinase/AKT; Prostate cancer; Metastasis; TGF β isoforms

Introduction

Transforming growth factor- β (TGF β) is a secreted cytokine involved in the regulation of many cellular processes and has been implicated as a factor in cancer formation and progression [1, 2]. TGF β exerts pleiotropic effects on numerous cellular functions that include differentiation, proliferation, motility and extracellular matrix (ECM) production in many normal cell types [3, 4]. In normal epithelial cells and early stage cancers, TGF β inhibits cell proliferation and acts as a tumor-suppressor [1, 5, 6]. However, in later stages of many cancers, the tumor cells become resistant to growth inhibitory effects of TGF β . In these cells, TGF β can act as a tumor promoter due to its role in stimulating angiogenesis, epithelial to mesenchymal transformation (EMT), or promoting the degradation of ECM; all of which aid in invasion and metastasis [2, 7, 8].

The three TGF β isoforms, TGF β 1, TGF β 2 and TGF β 3, share a 70–80 % sequence homology in most organisms and are expressed throughout most mammalian tissues [3, 4, 9]. Of the three isoforms, TGF β 1 is the most commonly studied and tends to be ubiquitously expressed among cell lines and in diseased tissues such as cancer [10, 11]. In numerous in vitro studies, all three isoforms bind to the same receptors and exert similar biological effects on target cells [12]. However, TGF β isoforms can have non-redundant specific effects during development as indicated by gene knock-out studies [13–16]. Binding affinity studies show that the three isoforms signal by binding to TGF β RI and TGF β RII [17] however, TGF β 2 differs from TGF β 1 and TGF β 3 in that it binds to TGF β RII with an affinity that is 100–1,000 fold lower and requires TGF β RIII (β -glycan) to promote receptor assembly with TGF β 2 [9, 18–22]. Additionally, crystal structure of TGF β RII-TGF β 3 complex showed that interfacial residues of TGF β 3 are identical to TGF β 1 but are different than TGF β 2 at three positions [23]. These studies suggest that although both TGF β 1 and TGF β 3 share a significant homology, receptor binding affinity and can exert similar biological effects on target cells, they may retain the ability to have non-redundant differential effects.

Earlier studies have indicated that individual TGF β isoforms may exert differential effects on cancer cells during different stages of the disease. In one such study, TGF β 3 increased the invasiveness of endometrial carcinoma cells via a PI3-kinase-dependent pathway, and these effects were distinct from those of TGF β 1 [24]. In prostate cancer, there is very little known about the expression levels of the three isoforms, specifically TGF β 3, in prostate cancer cell lines and clinical tissue samples. Evidence indicates that TGF β 3 expression increases 2 fold in prostate tumors and that the TGF β 3 gene is over-expressed in an androgen-independent derivative of prostate cancer cell line compared to the androgen-dependent parental cell line [25].

Recent studies have suggested the involvement of the phosphatidylinositol 3-kinase (PI3-kinase) pathway in TGF β -mediated effects on cancer cell invasion and metastasis. PI3-kinase has been shown to play an integral role in many cellular processes including cell proliferation, growth and survival in both normal and diseased tissues [26, 27]. Activating mutations of the PI3-kinase pathway and loss of PTEN are common in many types of cancers and are associated with tumor progression [28]. It has been shown that TGF β can activate PI3-kinase, as determined by increased phosphorylation of AKT, a downstream target of PI3-kinase [29–33]. Prior studies have also revealed that TGF β isoform-specific effects on the PI3-kinase pathway can be either dependent or independent of Smad signaling depending on the experimental conditions and cellular context [24, 32, 33]. The PI3-kinase pathway has also been implicated as a contributing pathway to TGF β induced EMT as well as fibroblast proliferation and morphological transformation [33], all precursors to invasion and metastasis. Whether or not TGF β isoforms play a differential role in invasion and metastasis of prostate cancer, and act through non-Smad pathways such as PI3-kinase, is still unclear.

In the present study, we have carried out a detailed analysis of the expression of TGF β isoforms and signaling components in cell line models representing different stages of prostate cancers and have studied the differential effects of specific isoforms on migratory and invasive behavior of prostate cancer cells. Our results indicate that TGF β effects on migration and invasion of prostate cancer cells are primarily induced by TGF β 3 and that these effects are dependent on both TGF β RI and Smad3 and mediated via PI3-kinase pathway.

Materials and methods

Chemicals and reagents

Recombinant human TGF β 1 and TGF β 3 were purchased from R&D systems (Minneapolis, MN). Inhibitors of TGF β RI (SB431542) and Smad3 (SIS3) were purchased from Tocris Bioscience (Ellisville, MO) and EMD Biosciences (Gibbstown, NJ), respectively. Specific inhibitor of PI3-kinase (LY294002) was purchased from Sigma-Aldrich (St. Louis, MO). The antibodies against pAKT-ser473, AKT (pan), pSmad2, pSmad3 and Smad2/3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against TGF β 1, 2 and 3 were purchased from Peprotech (Rocky Hill, NJ). Anti- β -Actin (clone AC-15) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-rabbit IgG HRP was purchased from Bio Source (Camarillo, CA) and Anti-mouse IgG HRP was obtained from Promega (Madison, WI). Matrigel and invasion inserts were purchased from BD Biosciences (Bedford, MA). The HEMA 3 stat dye was purchased from Fisher (Pittsburgh, PA).

Cell lines and cell culture

Prostate cell lines LNCaP (prostate carcinoma), DU145 (prostate carcinoma), PC3 (prostate adenocarcinoma), RWPE1 (epithelial cells from histologically normal adult human prostate), RWPE2 (immortalized epithelial cells were derived from RWPE-1 cells by transformation with Ki-ras using the Kirsten murine sarcoma virus (Ki-MuSV)) and PZ-HPV7 (immortalized epithelial cells from histologically normal adult human prostate) were acquired from American Type Cell Culture Collection (Rockville, MD). PC3M cells (metastatic derivative of PC3) were kindly provided by Dr. Girish Shah (University of Louisiana). All cells were cultured in recommended growth medium at 37°C with 5 % CO₂ and 100 % humidity. DU145, PC3, and PC3M cells were cultured in Eagle's minimum essential medium (MEM) with Earle's salts with 0.1 mM of the necessary amino acid supplements as described previously [34, 35]. LNCaP cells were maintained in RPMI 1640 containing 4 mM glutamine and 50 μ g/ml gentamycin. Both MEM and RPMI media (Mediatech, Herndon, VA) were supplemented with 5 % fetal bovine serum (FBS; HyClone, South Logan, Utah). RWPE1, RWPE2 and PZ-HPV7 were cultured in keratinocyte serum free medium (KSFM) containing 50 μ g/ml gentamycin (Invitrogen) and with 0.05 mg/ml bovine pituitary extract and 5 ng/ml EGF.

RNA isolation, cDNA synthesis and RT-PCR

For RT-PCR analysis, cells were seeded (5×10^5 /well) into 6-well plates overnight. Total RNA was isolated from the cells using TRIzol (Invitrogen) followed by chloroform extraction and isopropanol precipitation and quantified by optical density reading at 260 nm. OD₂₆₀/OD₂₈₀ ratios were used for quality assessment of RNA preparations. Total RNA (2 μ g) were reverse transcribed as previously described [36]. RT-PCR reactions were performed on I-Cycler IQ (BioRad, Hercules, CA). RT-PCR reactions were performed according to procedures described previously [34]. Gene encoding ribosomal protein L-19 was used as internal control. All gene-specific primers were designed with the assistance of the computer program Beacon Designer 5.0 (PremierBiosoft International, Palo Alto, CA) which was set to exclude regions of cross-homology against the non-redundant set of human genes from GenBank, EMBL, and DDBJ database sequences) and to exclude regions of significant secondary RNA structure. When possible, primer sets were designed to span long introns to avoid amplification of genomic DNA contamination. Sequences of all primers used in RT-PCR experiments are shown in Table 1. The PCR products were visualized on 1–2 % agarose gels stained with ethidium bromide or on 4 % SFR agarose gels (Amresco, Solon, OH). Analyses of PCR products were carried out in three independent experiments using different cell preparations.

TGF β treatments

To determine the effects of TGF β isoforms on phosphorylation of AKT, DU145, PC3, and LNCaP cells were cultured in 6 well plates (5×10^5 cells/well) in 5 % FBS/Medium and allowed to attach overnight. Cells were serum starved for 2 h and incubated with or without TGF β RI inhibitor (SB431542: 5 μ M), Smad3 inhibitor (SIS3: 3 μ M) or PI3-kinase inhibitor (LY294002: 10 μ M) for 30 min. Phosphatase inhibitor (Sodium Vanadate; 200 μ M) was added 10 min prior to the treatment. Cells were then treated with TGF β 1 or TGF β 3 (5 ng/ml) over various time points. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Sodium Vanadate, 1 μ g/ml leupeptin and 1X protease inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentrations were determined by the Lowry HS assay using the Bio-Rad DC Protein Assay kit according to the instructions provided by the manufacturer.

Western blot analyses

Cell lysates were mixed with Laemmli buffer (62.5 mM Tris, pH 6.8, 2 % SDS, 5 % β -mercaptoethanol and 10 % glycerol) and individual samples (25–50 μ g proteins) were subjected to SDS-PAGE in 8–10 % gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h in TBST (50 mM Tris, pH 7.5, containing 0.15 M NaCl, 0.05 % Tween 20) containing 5 % fat free skim milk. The blots were then incubated with appropriate dilutions of specific primary antibodies overnight at 4 °C in TBST containing 5 % bovine serum albumin (BSA). After washing, blots were incubated with anti-rabbit or anti-mouse immunoglobulins coupled to horseradish peroxidase (dilution 1:10,000) in blocking buffer (TBST with 5 % milk) for 1 h and washed in TBST for 1 h. The blots were developed in SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) or ECL Prime (Amersham) for 3 min, exposed to an X-ray film and visualized by autoradiography. Western blots for AKT (pan), Smad2/3 and β -actin were carried out in parallel as loading controls. The relative intensities of specific protein bands were determined by QuantityOne image analysis software.

Scratch wound assay

The migratory properties of DU145, PC3, and LNCaP cells were measured using a scratch wound assay. Cells were plated in 6-well plates (5×10^5 cells/well) in MEM with 5 % FBS and cultured overnight. Before treatment, wells were scratched down the middle with a 200 μ l pipette tip. Culture media were replaced with MEM containing 0.1 % FBS and cells were treated with TGF β 1 or TGF β 3 (1 ng/ml) with or without specific inhibitors of TGF β RI (SB431542: 5 μ M), Smad3 (SIS3: 3 μ M) or PI3-kinase (LY294002: 10 μ M). Cells were allowed to migrate across the scratch for 48 h. Images of the scratch area were recorded at three random spots at 0 and 48 h. The migrating cells were counted using a standard size field for each image. Statistical analysis was performed using students *t* test ($n = 3$) with SigmaPlot Analysis Software.

Invasion assay

The invasive properties of DU145 and PC3 were measured using the BD BioCoat Matrigel Invasion inserts. Inserts (BD Biosciences) were coated with 50 μ l of a 1:4 Matrigel/Medium dilution (BD Biosciences) and allowed to solidify at 37 °C for 1 h. Cells were resuspended (5×10^4 cells/ml) in MEM with 0.1 % FBS and 500 μ l of cell suspension was added to each insert. Cells were treated with or without specific inhibitors of TGF β RI (SB431542: 5 μ M), Smad3 (SIS3: 3 μ M) or PI3-kinase (LY294002: 10 μ M) followed by TGF β 1 or TGF β 3 (0.1, 1 or 10 ng/ml) and allowed to invade through a porous membrane coated with Matrigel

at 37 °C for 48 h. Matrigel and non-invading cells were removed via scrubbing. Invading cells on the membrane were fixed in 3.7 % paraformaldehyde and stained using the HEMA3 stain set (Fisher). Pictures were taken in five different fields for average number of invading cells to be determined. Statistical analyses were performed using students *t* test ($n = 4$) with SigmaPlot Analysis Software.

MTT assay

PZ-HPV7, DU145 and PC3 cells were used as target cells for the detection of any differences in the bioactivity of recombinant TGF β isoforms using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega) [37, 38]. Cells were seeded (5×10^3 cells/well) in 96-well plates in recommended growth media. After allowing cells to attach overnight, medium was replaced and recombinant TGF β 1 or TGF β 3 were added at the concentration of 1 and 10 ng/ml and incubated for 48 h at 37 °C. After 48 h, medium was again replaced and 15 μ l Dye Solution added and incubated at 37 °C in the dark for 4 h. Following 4 h incubation, solubilization/stop solution was added and incubated for 1 h. Absorbance was read at 570 nm using a 96-well plate reader. Three independent experiments were carried out with similar results.

Results

Gene and protein expression of TGF β isoforms, receptors and Smad proteins in prostate cell lines

Gene expression of TGF β isoforms and receptors in prostate cells was determined by RT-PCR across several established cell lines, with L-19 used as a control (Fig. 1a). TGF β 1 mRNA was expressed across all seven cell lines (PZ-HPV7, RWPE1, RWPE2, DU145, PC3, PC3M and LNCaP); however the expression was substantially lower in LNCaP cells. TGF β 2 mRNA was detected in six of the seven cell lines with low expression in PZ-HPV7, RWPE1 and RWPE2 cells. A slight increase was detected in cancer cell lines DU145, PC3 and PC3M, however there was no detectable expression of TGF β 2 in LNCaP cells.

TGF β 3 expression was relatively low in normal prostate epithelial cell line PZ-HPV7 and in LNCaP cells, and was slightly higher in RWPE1 and RWPE2 cells, as well as in DU145 cells. The highest expression of TGF β 3 was found in PC3 and PC3M cells, both metastatic prostate cancer cell lines.

TGF β RI mRNA was expressed throughout all seven cell lines, with LNCaP cells again having the lowest expression. TGF β RII was expressed in PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells; however it was not detected in LNCaP cells. TGF β RIII was detected at low levels in PZ-HPV7, RWPE1, RWPE2, DU145, PC3, and PC3M cells, however it was not detected in LNCaP cells. The mRNA expression of Smad2 and Smad3 (R-Smads), Smad4 (Co-Smad) and Smad7 (I-Smad) were detected in all seven cell lines.

Western blot analysis was performed to determine relative protein abundance of TGF β isoforms (TGF β 1, 2 and 3) in total cell lysates from PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells (Fig. 1b) and for TGF β RII in cancer cell lines LNCaP, DU145 and PC3 cells (Fig. 1c). All cells were cultured under standard growth conditions. TGF β 1 (50kd precursor protein) was expressed across all cell lines, while TGF β 2 (25kd mature protein) was detected only in PC3 and PC3 M cells. TGF β 3 (50kd precursor protein) was detected in all cell lines, with high levels in metastatic cell lines DU145, PC3 and PC3M cells. TGF β RII protein was detected in DU145 and PC3 cells, however it was absent in LNCaP cells. LNCaP cells were included in future assays as a negative control to rule out non-specific effects of TGF β isoforms as LNCaP cells do not contain mRNA or protein signaling TGF β RII.

These studies showed an increase in both gene and protein expression of TGF β 3 in metastatic cell lines DU145 and PC3 cells suggesting a possible autocrine role of this isoform in migration and invasion. Therefore, for further studies, DU145 and PC3 cell lines were selected to determine migratory and invasive potential in response to exogenous TGF β 1 and TGF β 3. Both DU145 and PC3 cells express all of the TGF β ligands, receptors and Smad proteins.

Differential effects of TGF β isoforms on proliferation, migration and invasion of prostate cancer cells

TGF β is known to inhibit proliferation in normal prostate epithelial cells [39]. The effects of TGF β 1 and TGF β 3 on the proliferation of immortalized normal epithelial (PZ-HPV7) cells and prostate cancer cell lines DU145 and PC3 were determined using MTT assay in order to rule out possible differences in the biopotencies of recombinant proteins. Figure 2a shows that both TGF β 1 and TGF β 3 significantly inhibited PZ-HPV7 and DU145 cell proliferation at both 1 and 10 ng/ml, with no differences in the potencies of the two isoforms. PC3 cells did not respond to the inhibitory effects of either isoform.

The effects of TGF β 1 and TGF β 3 on migration of metastatic prostate cancer cell lines PC3, DU145 and LNCaP were determined using a scratch wound assay. PC3, DU145 and LNCaP cells were treated with either TGF β isoform and allowed to migrate according to established procedures. As shown in Fig. 2b, both TGF β isoforms caused an increase in the migration of PC3 cells. However, TGF β 3 effects were 2.1 fold ($n = 3$) higher than those of TGF β 1 on number of migrating cells (See Fig. 3b; $P < 0.01$). Both TGF β isoforms had no effects on the migration of DU145 and LNCaP cells under similar experimental conditions.

As shown in Fig. 2c, both TGF β 1 and TGF β 3 increased invasiveness of PC3 cells. TGF β 1 had a significant effect on invasion at 0.1 ng/ml ($P < 0.05$), which declined at both 1 and 10 ng/ml doses. However, TGF β 3 caused significantly higher invasion of PC3 cells compared to TGF β 1 at 1 ng/ml ($P < 0.05$) and 10 ng/ml ($P < 0.01$) doses (Fig. 2d). Both TGF β isoforms had no effect on invasiveness of DU145 cells under identical experimental conditions.

The role of TGF β RI and Smad3 on TGF β isoform induced migration and invasion

To determine whether TGF β 1 and TGF β 3 effects on cell migration and invasion are mediated by classical TGF β signaling pathway, PC3 cells were treated with TGF β 1 or TGF β 3 in the presence of specific inhibitors of TGF β RI and Smad3. As shown in Fig. 3a, b, both TGF β RI inhibitor (SB431542) and Smad3 Inhibitor (SIS3) blocked TGF β 1 and TGF β 3 induced migratory effects on PC3 cells. TGF β RI inhibitor (SB431542) and Smad3 inhibitor (SIS3) also blocked the effects of TGF β isoforms on invasive behavior of PC3 cells (Fig. 3c, d).

Differential effects of TGF β isoforms on migration and invasion are mediated by PI3K pathway

To determine if TGF β isoform-induced migration and invasion is mediated via the PI3-kinase pathway, scratch wound and invasion assays were performed in the presence or absence of a specific PI3-kinase inhibitor (LY294002: 10 μ M). PC3 Cells were treated with TGF β 1 or TGF β 3 (1 ng/ml) and allowed to migrate across a scratch or through a matrigel-coated membrane for 48 h. PI3-kinase inhibitor (LY294002) blocked both TGF β 1 and TGF β 3 induced migratory behavior in PC3 cells (Fig. 3a, b). PI3-kinase inhibitor (LY294002) also blocked the effects of both isoforms on the invasion of PC3 cells (Fig. 3c, d).

Differential effects of TGF β isoforms on activation of PI3-kinase pathway

To determine if TGF β isoforms differentially activate the PI3-kinase pathway and phosphorylation of AKT (pAKT), PC3 cells were treated with either TGF β 1 or TGF β 3 (5 ng/ml) for specific time points and probed for pAKT^{ser473}, a downstream target of PI3-kinase. Both TGF β isoforms induced an increase in the phosphorylation of AKT^{ser473} over several time points in PC3 cells. TGF β 3 was more effective than TGF β 1 in increasing pAKT^{ser473} (data not shown). We then compared TGF β isoform induced pAKT^{ser473} in DU145, PC3 and LNCaP cells 15 and 60 min after treatment. TGF β isoforms again induced an increase in the phosphorylation of AKT^{ser473} in PC3 cells (Fig. 4a, b). Once again, TGF β 3 was more effective at inducing the phosphorylation of AKT^{ser473} with a significant (3.7 fold; $n = 3$) increase at 60 min as determined by densitometry using β -actin as a control. However, neither TGF β isoform increased pAKT^{ser473} levels in DU145 or LNCaP cells at both time points.

To determine whether TGF β 1 and TGF β 3 induced pAKT^{ser473} are mediated by TGF β RI, Smad3 or PI3-kinase pathway, PC3 cells were treated with a TGF β RI inhibitor (SB431542), Smad3 inhibitor (SIS3) or PI3-kinase inhibitor (LY294002) followed by TGF β 1 or TGF β 3 (5 ng/ml). Cell lysates were collected and analyzed for pAKT^{ser473}, Total AKT (pan) and β -actin. Both TGF β RI inhibitor (SB431542) and Smad3 inhibitor (SIS3) blocked TGF β -induced pAKT^{ser473} (Fig. 5a). PI3-kinase inhibitor (LY294002) also blocked basal and TGF β -induced accumulation of pAKT^{ser473} in PC3 cells. We also looked at the phosphorylation of Smad 2 and Smad3 upon treatment with the same inhibitors (Fig. 5b). SB431542 blocked the effects of both TGF β 1 and TGF β 3 on phosphorylation of Smad2 and Smad3. SIS3 blocked effects of both isoforms on Smad3 phosphorylation without affecting the phosphorylation of Smad2. LY294002 did not influence Smad2 or Smad3 phosphorylation in response to both isoforms.

Discussion

This study is the first of its kind to systematically determine the expression of all TGF β isoforms, receptors and Smads simultaneously in several prostate cell lines. Though previous studies have shown the presence of TGF β isoforms and machinery in different cancers including prostate tissues and cell lines, none have looked at the relative mRNA and protein expression levels in numerous prostate cell lines under the same experimental conditions. We have shown that TGF β 1 is ubiquitously expressed at both the mRNA and protein level in both normal and metastatic prostate cell lines. This suggests that any differential effects seen in the normal versus metastatic cell lines may be due to changes in expression of other TGF β isoforms. Alternatively, post translation activation may play a role in changing effects of TGF β 1 during different stages of prostate cancer. We show an increase of both TGF β 2 and TGF β 3 in metastatic prostate cell lines, however TGF β 2 protein expression changes were only seen in PC3 cells. The steadiest change was seen in TGF β 3 expression, with it increasing consistently in the more metastatic cell lines. This may indicate a specific role of TGF β 3 in the meta-static phenotype. This differential isoform role is supported by similar high expression of TGF β 3 in breast carcinomas correlating with decreased overall survival rate [40, 41] and that TGF β 3 increased invasiveness of endometrial carcinoma cells [24].

Based on our expression data, we compared the effects of TGF β 1 and TGF β 3 on the motility of three metastatic prostate cancer cell lines, DU145, PC3 and LNCaP cells. We show that both DU145 and PC3 cells express TGF β 1 and TGF β 3 as well as all receptors and Smad proteins, whereas LNCaP lacks the TGF β RII, a necessary receptor for TGF β signaling. In addition, both TGF β isoforms induce phosphorylation of Smad2/3 in both cell lines to the same extent (Unpublished data). However, while both TGF β isoforms induced

migratory and invasive behavior in PC3 cells, DU145 and LNCaP cells were unresponsive to both isoforms under identical experimental conditions. Our ongoing studies on the effects of TGF β on cell proliferation show opposite results. While TGF β inhibits proliferation in DU145 cells, it has no effect on proliferation of PC3 cells (Strong et al., unpublished data). TGF β is known to exert differential effects depending on the context of the cell. These two cell lines may represent a model of the proposed TGF β “switch” from its tumor suppressor to tumor promoter activities.

We also show that both TGF β 1 and TGF β 3 increase migration and invasion of PC3 cells; however, TGF β 3 is significantly more potent than TGF β 1. These differences do not appear to be a consequence of differential bioactivities of the two recombinant proteins, since no differences were observed in the effects of the two isoforms on proliferation of normal prostate epithelial derived PZ-HPV7 cells and prostate cancer cell lines DU145 and PC3. The differential and more potent effects of TGF β 3 on invasive behavior of prostate cancer cells are similar to those previously reported on TGF β 3 induced invasiveness of endometrial carcinoma cells [24]. Prior studies have also revealed that TGF β isoform-specific effects can be both dependent or independent of Smad signaling depending on the context in which it is studied [24, 32, 33]. Our study indicates that the TGF β isoform-specific increase of migration and invasion is TGF β RI and Smad3 dependent.

The PI3-kinase pathway has been shown to play an essential role in the migration and invasion of many cell types. Previous studies indicate that TGF β can activate PI3-kinase, as shown by increased phosphorylation of AKT [29–33], leading to increased invasion of cancer cells [24]. In keeping with these studies, our results indicate that the PI3-kinase pathway is essential for both TGF β 1 and TGF β 3 induced migration and invasion. Pharmacological inhibition of PI3-kinase blocks TGF β isoform-induced movement of PC3 cells. Both isoforms cause an increase in the phosphorylation of AKT in PC3 cells, with TGF β 3 being more potent than TGF β 1. This TGF β isoform-induced phosphorylation of AKT is both TGF β RI and Smad3 dependent. Our results also indicate that Smad2 may not mediate these effects of TGF β isoforms on AKT phosphorylation and invasive behavior.

These results, along with previous studies, confirm an isoform-specific role of TGF β in the migration and invasion of the metastatic PC3 prostate cancer cells which are dependent on the activation of the PI3-kinase pathway. We did not see similar effects of TGF β isoforms on activation of PI3-kinase and AKT phosphorylation in DU145 and LNCaP cells, indicating that lack of this response may be responsible for lack of TGF β effects on migration of these cells. It is tempting to speculate that the ability of TGF β to activate PI3-kinase signaling may be pre-requisite for the induction of invasive behavior in more advanced stages of prostate cancers and may represent the “switch” from anti-tumorigenic to tumor-promoter role of TGF β .

One possible contributor of differential effects of TGF β on activation of PI3-kinase DU145, LNCaP and PC3 cells may be the Phosphatase and Tensin Homolog (PTEN), which inhibits PI3-kinase dependent phosphorylation of AKT. PTEN has been shown to be mutated in many cancers which can lead to higher basal levels of pAKT^{ser473} and increased survival of cells [28, 42, 43]. PTEN is active in the DU145 cell line, but is mutated and biologically inactive in PC3 cells and absent from LNCaP cells altogether [44–47]. Although LNCaP cells have higher basal levels of pAKT, we did not observe any effects of TGF β on pAKT^{ser473}, due to lack of TGF β signaling in these cells. It is therefore possible that PTEN plays a role in TGF β effects on activation of PI3-kinase in prostate cancer cells and that TGF β dependent invasive and metastatic behavior may be more pronounced in patients where the tumor cells harbor PTEN mutations. This suggests that PTEN and, ultimately, the

PI3-kinase pathway may have a role in the TGF β isoform-induced migration and invasion and the TGF β “switch” from anti- to pro-tumorigenic cytokine in prostate cancer.

In conclusion, we show an increased expression of TGF β 3 in cell line models of advanced prostate cancer and that this isoform is more potent than TGF β 1 in activation of PI3-kinase signaling pathway and invasive behavior in these cells. We also show that the effects of TGF β isoforms on cell migration and invasion are mediated by both TGF β RI and Smad3 dependent activation of the PI3-kinase pathway and therefore simultaneous increase in TGF β 3 expression and signaling and PTEN mutations may result in increased metastatic behavior of prostate cancer cells.

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Fig. 1.

Expression of TGFβ isoforms and signaling components in prostate cell lines; **a** Semi-quantitative RT-PCR was performed using RNA from PZ-HPV7, RWPE1, RWPE2, DU145, PC3, PC3M, and LNCaP cells to determine relative mRNA levels of TGFβ isoforms (TGFβ1, 2 and 3), TGFβ receptors I, II and III and Smads 2, 3, 4 and 7. The mRNA levels in all cell line samples were normalized against L-19, which served as an internal control. **b** Western blot analysis was performed to probe for TGFβ isoforms (TGFβ1, 2 and 3) in total cell lysates from PZ-HPV7, RWPE1, RWPE2, DU145, PC3 and PC3M cells. **c** Western blot analysis was performed to determine relative protein levels of TGFβRII in LNCaP, DU145 and PC3 cells. Western blots for β-actin were performed as protein loading controls

Fig. 2.

Effects of TGF β isoforms on cell proliferation, migration and invasion of prostate cell lines. **a** Dose dependent effects of TGF β 1 and TGF β 3 on proliferation of PZ-HPV7, DU145 and PC3 cells. Each bar represents Mean $\pm$ SD from a representative experiment of three independent experiments. **b** Effects of TGF β 1 and TGF β 3 on migration of PC3, DU145 and LNCaP prostate cancer cells. The migratory properties of PC3, DU145 and LNCaP cells were measured using a scratch wound assay. Cells were cultured overnight and scratched with a 200 μ l pipette tip. Culture media were replaced with MEM or RPMI (containing 0.1 % FBS) and the cells were treated with TGF β 1 or TGF β 3 (1 ng/ml) for 48 h. Images of the scratch area were recorded at three random spots at 0 and 48 h. The migrating cells were counted using a standard size field for each image and the Mean $\pm$ SEM from three independent experiments is shown in the insert at the lower panel. Statistical analysis was performed as shown in Fig. 3b. **c** The invasive properties of DU145 and PC3 cells were measured using the BD BioCoat Matrigel Invasion inserts. Cells were treated with different concentrations of TGF β 1 or TGF β 3 and allowed to invade through a porous membrane coated with Matrigel for 48 h. Pictures were taken in five different fields for average number of invading cells to be determined. **d** Number of invading PC3 cells through Matrigel after treatment with TGF β 1 or TGF β 3 for 48 h. Each bar represents Mean $\pm$ SEM from 4 independent experiments. *Significantly different ($P < 0.05$; Students t test) from appropriate controls

Fig. 3.

The effect of TGF β 1 (1 ng/ml) and TGF β 3 (1 ng/ml) on migration and invasion of PC3 cells in the presence of inhibitors of TGF β RI (SB431542), Smad3 (SIS3) and PI3-kinase (LY294002). **a** Scratch wound assays were performed following treatment with TGF β isoforms for 48 h in the presence or absence of specific inhibitors. **b** Number of migrating cells after various treatments. Each bar represents Mean $\pm$ SEM from three independent experiments. *a* statistically significant ($P < 0.05$) from untreated controls, *b* statistically significant ($P < 0.05$) from TGF β 1 treated cells, *c* statistically significant ($P < 0.05$) from TGF β 3 treated cells. **c** Invasion assays were performed following treatment with TGF β isoforms for 48 h in the presence or absence of specific inhibitors. **d** Number of invading cells after various treatments. Each bar represents Mean $\pm$ SEM from three independent experiments. *a* statistically significant ($P < 0.05$) from untreated controls, *b* statistically significant ($P < 0.05$) from TGF β 1 treated cells, *c* statistically significant ($P < 0.05$) from TGF β 3 treated cells

Fig. 4. The effects of TGFβ1 and TGFβ3 on phosphorylation of AKT^{ser473} in prostate cancer cells. **a** Comparative effects of TGFβ1 and TGFβ3 on phosphorylation of AKT^{ser473} in PC3, DU145 and LNCaP cells after treatment with the two isoforms for 15 and 60 min. Total (pan) AKT and β-actin were used as loading controls. Data is presented from a representative experiment. **b** Band density analysis of pAKT^{ser473} in PC3 cells after treatment with TGFβ isoforms for 15 or 60 min. Each band density was normalized by density of β-actin bands. Each *bar* represents Mean ± SE from three independent experiments. *Significantly different ($P < 0.05$) from untreated controls

Fig. 5. Effects of TGF $\beta 1$ and TGF $\beta 3$ on phosphorylation of AKT^{ser473}, Smad2 and Smad3 in PC3 cells in the presence or absence of inhibitors of TGF β RI (SB431542), Smad3 (SIS3), or PI3-kinase inhibitor (LY294002). **a, b** PC3 cells were incubated with specific inhibitors for 30 min followed by treatment with TGF $\beta 1$ or TGF $\beta 3$ (5 ng/ml) for 60 min. Cells treated with EGF (5 ng/ml) were used as positive controls. Total (pan) AKT and β -actin were used as loading controls

Table 1

Gene-specific primers used for RT-PCR amplification

Gene	Forward primer	Reverse primer	Product size (bp)
TGF β 1	5'-CATCAGAGCTCCGAGAAGCG-3'	5'-GCTAAGGCGAAAGCCCTCA-3'	250
TGF β 2	5'-ACAAAATAGACATGCCGCC-3'	5'-GCTGTCGATGTAGCGCTGG-3'	250
TGF β 3	5'-TGTCACACCTTTCAGCCAA-3'	5'-TCCAAGTTGCGGAAGCAGT-3'	250
TGFR1	5'-CGTTCGTGGTTCCGTGAG-3'	5'-CAATGGTATCTGTGGCTGAAT-3'	378
TGFR2	5'-GATGCTGCTTCTCCAAAGTG-3'	5'-TCTGTGTTGTGGTTGATGTTG-3'	376
TGFR3	5'-CTAAGTGTGTGCCTCTGACG-3'	5'-CAAACGCAATGCCCATCACG-3'	216
Smad2	5'-CTTTTGTGTGTAAGCTCTACTG-3'	5'-GACCTTCTACCACTTTCAGAGTTG-3'	247
Smad3	5'-GGGATACCAGCAGCAAGAGAG-3'	5'-ATTGTGAAGACGTTACCTGAAAG-3'	201
Smad4	5'-TTGCGTCAGTGCATCGACAG-3'	5'-CCAGCCTTTCACAAACTCATCC-3'	209
Smad7	5'-AATATTTCTCTCTGAGTGCTTGC-3'	5'-ATTCTGCTTCCCCTCTTCTATC-3'	222
L19	5'-GAAATCGCCAATGCCAACTC-3'	5'-TCTTAGACCTGCGAGCCTCA-3'	405