Transforming Growth Factor-β1 Modulates Tumor-stromal Cell Interactions of Prostate Cancer through Insulin-like Growth Factor-I

MANABU KAWADA, HIROYUKHI INOUE, MASAYUKI ARAKAWA and DAISHIRO IKEDA

Drug Development Unit, Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, 18-24 Miyamoto, Numazu-shi, Shizuoka 410-0301, Japan

Abstract. *Insulin-like growth factor-I (IGF-I) secreted from the prostate stroma mediates tumor-stromal cell interactions in prostate cancer development. We have recently reported that human prostate stromal cells (PrSC) stimulate human prostate cancer DU-145 cell growth via IGF-I. Transforming growth factor-β1 (TGF-β1) also plays a critical role in tumor-stromal cell interactions, but TGF-β1 has pleiotropic effects as it can modulate growth of prostate cancer either positively or negatively. To elucidate the complex behavior of TGF-β1, the effect of TGF-β1 on the IGF axis in PrSC was studied. TGF-β1 increased the expression of IGF-I and IGF binding protein-3 (IGFBP-3). RT-PCR experiments revealed that TGF-β1 up-regulated the mRNA expression of IGF-I and IGFBP-3. However, while TGF-β1 significantly increased IGFBP-3 secretion by PrSC, it conversely reduced the amounts of biologically active IGF-I unbound to IGFBP-3. Immunohistochemical analyses of 49 human prostate cancer tissues showed that IGF-I expression in the stroma correlated positively with TGF-β1 expression in the stroma ($r=0.551$, $p=0.0001$). Furthermore, TGF-β1 actually suppressed the growth of DU-145 cells in coculture with PrSC. But, IGFBP-3 proteinases, such as prostate specific antigen (PSA) and matrix metalloproteinase-7 (MMP-7), restored the DU-145 cell growth, suggesting that degradation of IGFBP-3 potentiates cancer growth. Taken together, these results indicated that TGF-β1 modulates the growth of prostate cancer, either positively or negatively, through the balance between the amounts of IGF-I and IGFBP-3. This complex behavior of TGF-β1 on the IGF axis is one explanation for the pleiotropic activities of TGF-β1 on the growth of prostate cancer.*

Correspondence to: Manabu Kawada, Drug Development Unit, Numazu Bio-Medical Research Institute, Microbial Chemistry Research Center, 18-24 Miyamoto, Numazu-shi, Shizuoka 410-0301, Japan. Tel: +81 55 924 0601, Fax: +81 55 922 6888, e-mail: kawadam@bikaken.or.jp

Key Words: Prostate cancer, prostate stroma, TGF-β, IGF-I, tumor growth.

Solid tumors are composed of tumor cells, as well as the surrounding stroma, including the extracellular matrix, fibroblasts, macrophages and endothelial cells (1). Because the growth of tumor cells is regulated by the stromal cells through their diffusible factors and adhesion (2-5), tumor-stromal cell interactions significantly contribute to the development of some carcinomas, such as those of the breast and prostate (6, 7). Many growth factors and cytokines are known to be involved in the regulation of tumor-stromal cell interactions (2, 8). The various lines of evidence have suggested the involvement of the insulin-like growth factor (IGF) axis in prostate cancer development (9, 10).

The IGF axis consists of two major ligands (IGF-I and IGF-II), two cell surface receptors (IGF-IR and IGF-IIR), and six binding proteins (IGFBP-1 to 6) that regulate IGF availability to the receptors and a group of IGFBP proteases that cleave IGFBP and modulate the action of IGFs (11-13). IGF-I binds to IGF-IR, and the tyrosine kinase of the cytoplasmic domain of IGF-IR transduces IGF-I signals into cells (11, 13, 14). Prostate cancer cells respond to the mitogenic actions of IGF-I (15, 16), and prostate cancer tissues show high expression of IGF-IR (17, 18). Several epidemiological studies have suggested that reduced plasma levels of IGFBP-3, increased plasma levels of IGF-I or an increased ratio of IGF-I to IGFBP-3 are associated with an increased risk for the development of several common carcinomas such as breast, prostate, lung and colon carcinomas (11, 14, 19). Because the prostate stroma secretes IGF-I (17), we investigated the significance of prostate stromal IGF-I in the regulation of prostate cancer growth. Using an *in vitro* assay and animal models, we recently reported that normal human prostate stromal cells (PrSC) increased the growth of human prostate cancer DU-145 cells in coculture *via* IGF-I secreted by the PrSC (20, 21). Thus the local secretion of IGF-I in prostate stroma plays a critical role in prostate cancer development as

well as circulating IGF-I (21), promoting the idea that IGF-I signaling is a viable target for the treatment of prostate cancer.

The stroma that regulates tumor growth through tumor-stromal cell interactions is designated reactive stroma or cancer-associated fibroblasts (2, 8). The reactive stroma expresses vimentin and smooth muscle α-actin (SMA) indicating a myofibroblast phenotype (22). Myofibroblasts express various molecules that regulate tumor growth and transforming growth factor-β (TGF-β) is a well-known inducer of myofibroblasts. TGF-β stimulates fibroblasts, including PrSC, to differentiate into myofibroblasts (22-24) and to produce growth factors, extracellular matrix components and matrix remodeling enzymes for tumor progression (25). Thus, TGF-β is one regulator of the reactive stroma that plays a critical role in tumor-stromal cell interactions (2, 8).

TGF-β1 is a multipotent molecule that either suppresses or enhances tumor growth (25-28). The expression of TGF-β1 is higher in tumor tissues, such as prostate and breast carcinomas, than normal tissues (25, 29-31). Inhibition of TGF-β1 action resulted in the inhibition of prostate cancer growth in a mouse xenograft model (25). In contrast, the conditional inactivation of the TGF-β receptor resulted in increased prostate neoplasia in a mouse model (32, 33). These opposing results are thought to be due to the effect of TGF-β1 on the tumor environment, including the stroma and immune response, in addition to a direct action on tumor cells. To elucidate the complex behavior of TGF-β1 on prostate cancer, the effect of TGF-β1 on the IGF axis was studied and any correlation of IGF-I and TGF-β1 expressions was investigated in human prostate stromal cells (PrSC).

Materials and Methods

Reagents. Rhodanile blue was purchased from Aldrich (Milwaukee, WI, USA). Insulin and hydrocortisone were obtained from Sigma (St. Louis, MO, USA). Transferrin was obtained from Wako Pure Chemical Industries (Tokyo, Japan). The recombinant human basic fibroblast growth factor (bFGF), recombinant human TGF-β1, and recombinant human IGF-I were purchased from Pepro Tech (London, United Kingdom). The recombinant human IGFBP-3 and recombinant human matrix metalloproteinase-7 (MMP-7) were obtained from R&D Systems (Minneapolis, MN, USA) and the human prostate specific antigen (PSA) from Chemicon (Temecula, CA, USA). The antibodies used were: anti-vimentin and anti-SMA (Sigma); anti-IGF-I and anti-IGFBP-3 (R&D Systems); anti-IGF-IRβ (sc-713) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-phosphotyrosine (05-321) (Upstate Biotechnology, Lake Placid, NY, USA).

Cells. The human prostate cancer DU-145 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 100 units/ml penicillin G and 100 μg/ml streptomycin at 37˚C with 5% CO_2. The human normal prostate stromal cells (PrSC#1, 17-year-old organ donor; PrSC#2, 19-year-old organ donor) were obtained from Bio Whittaker (Walkersville, MD, USA) and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin, ITH (5 μg/ml insulin, 5 μg/ml transferrin and 1.4 μM hydrocortisone), and 5 ng/ml bFGF at 37˚C with 5% CO_2. Mouse normal fibroblasts NIH-3T3 overexpressing the human IGF-IR (NIH-IGF-IR) were constructed by transfecting NIH-3T3 fibroblasts with a mammalian expression vector of the human IGF-IR gene (IOH36576) (Invitrogen, Carlsbad, CA, USA), pcDNA6.2/DEST-IGF-IR, and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 25 μg/ml blasticidin.

Coculture experiment. A microplate assay method for the selective measurement of epithelial tumor cells in coculture with stroma cells using rhodanile blue dye was performed as previously described (20). The PrSC were first pipetted into 96-well plates at 5000 cells per well in 100 μl of DMEM supplemented with ITH and varying concentrations of FBS. After 2 days, 10 μl of prostate cancer cell suspension (5000 cells) in serum-free DMEM was pipetted onto a monolayer of PrSC, and the cells were further cultured for 3 days. For monoculture of prostate cancer cells, assay medium alone was first incubated for 2 days at 37˚C. Then prostate cancer cells were pipetted as described above, and cultured for further 3 days.

Measurement of IGF-I and IGFBP-3. The PrSC (10^5 cells) were pipetted into 12-well plates in 1 ml of DMEM supplemented with ITH and varying concentrations of FBS. The cells were cultured for 2 days in the presence or absence of 10 ng/ml TGF-β1 after which the cell culture supernatants were withdrawn. The amounts of IGF-I and IGFBP-3 were determined using human IGF-I and human IGFBP-3 Quantikine kits (R&D Systems).

Immunofluorescence. The PrSC ($5x10^4$ cells) were cultured on glass cover slips in DMEM supplemented with ITH and varying concentrations of FBS for 2 days in the presence or absence of 10 ng/ml TGF-β1. The cells were washed with phosphate-buffered saline (PBS) and fixed with cold acetone for 2 min. After blocking with 10% FBS-PBS for 20 min, the fixed cells were incubated with anti-vimentin (immunoglobulin M, IgM) in 1.5% FBS-PBS for 1 h, followed by anti-IgM Alexa Flour 488 (Molecular Probes, Eugene, OR, USA) in 1.5% FBS-PBS for 45 min. After washing with PBS, the fixed cells were further incubated with anti-SMA (IgG_2a) in 1.5% FBS-PBS for 1 h, followed by anti-IgG_2a Alexa Flour 546 (Molecular Probes) in 1.5% FBS-PBS for 45 min. The proportion of myofibroblasts (%) was calculated by dividing the number of vimentin- and SMA-double positive cells by the number of vimentin-positive cells.

Reverse transcription-PCR analysis. The PrSC ($2.5x10^5$) or DU-145 cells ($2.5x10^5$) were cultured in DMEM supplemented with ITH and varying concentrations of FBS for 2 days in the presence or absence of 10 ng/ml TGF-β1. The total RNA was isolated using the RNeasy Minikit (Qiagen, Hilden, Germany). The cDNAs were synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) with the same quantity of RNA (1 μg) and amplified using *Taq* DNA polymerase (Promega). The specific primers used were as follows: IGFBP-3 (158-bp), 5'-CGCCAGCTCCAGGAAATG-3' (sense) and 5'-GCTGTCTTT AGCATGCCCTTTCT-3' (antisense); SMA (590-bp), 5'-GCTCAC GGAGGCACCCCTGA-3' (sense) and 5'-CTGATAGGACATT GTTAGCAT-3' (antisense); IGF-I (342-bp) (34), IGF-II (359-bp) (34), IGF-IR (190-bp) (34), IGF-IIR (370-bp)

(34), IGFBP-2 (497-bp) (35), IGFBP-5 (370-bp) (35), IGFBP-6 (524-bp) (36), and GAPDH (246-bp) (37) have been reported elsewhere. The PCRs were optimized for each set of primers using different numbers of cycles to ensure that amplification occurred in a linear range. After amplification, the products were electrophoresed in a 2% agarose gel, stained with SYBR Green I (Cambrex Bio Science, Rockland, ME, USA) and analyzed with a FLA-5000 image analyzer (Fujifilm, Tokyo, Japan).

Preparation of conditioned medium. The PrSC ($5x10^5$ cells) were cultured in 10 ml of DMEM supplemented with 10% FBS and ITH for 2 days in the presence or absence of 10 ng/ml TGF-β1. Culture supernatants were collected and immunoprecipitated with the indicated antibodies as described previously (38). The IGF-I in the conditioned medium was blotted with a biotinylated anti-IGF-I antibody (R&D systems).

Immunohistochemistry. Tissue arrays of human prostate cancer were purchased from Folio Biosciences (Columbus, OH, USA). The tissue sections were deparaffinized in xylene and rehydrated in descending alcohols into water. Immunohistochemical staining was conducted with anti-IGF-I (R&D Systems), anti-IGFBP-3 (R&D Systemns), anti-TGF-β1 (R&D Systems), and anti-SMA (Lab Vision, Fremont CA, USA) using a mouse ABC staining system (Santa Cruz Biotechnology). The tissue sections were counterstained with hematoxylin. No significant staining was observed when the sections were incubated with the secondary antibody only. The estimated visual intensity of IGF-I, IGFBP-3, and TGF-β1 immunostaining was graded on an arbitrary three point scale: – (no staining); + (weak staining); and ++ (strong staining).

Degradation assay of IGFBP-3. IGF-I (1 μg/ml), IGFBP-3 (2 μg/ml), PSA (100 μg/ml), and MMP-7 (2 μg/ml) were incubated in a cleavage buffer (150 mM NaCl, 10 mM HEPES (pH 7.4), and 5 mM $CaCl_2$) for 2 h at 37˚C as described elsewhere (39). To activate the MMP-7, pro-MMP-7 was pre-incubated with 1 mM *p*-aminophenylmercuric acetate for 1 h at 37˚C. The reaction solution was used either for Western blotting for the detection of IGFBP-3 or the following procedure. NIH-IGF-IR cells ($3x10^5$) were cultured in DMEM supplemented with 1% FBS overnight. The medium was replaced with 0.9 ml of serum-free DMEM, after which 0.1 ml of the above reaction solution was added to the medium. After 30 min incubation at 37˚C, cell lysates were prepared and immunoprecipitated with an anti-IGF-IR antibody as described previously (38).

Statistical analysis. All the data were from two or three independent experiments. Statistical analysis was conducted by Student's *t*-test and Spearman's rank correlation test using StatView software (Abacus Concepts, Berkley, CA, USA). A *p*-value less than 0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of normal human prostate stromal cells (PrSC). Because this study employed two PrSCs derived from two different donors, the characteristics of these PrSCs (PrSC#1 and PrSC#2) were verified. As shown in Figure 1a, at low concentrations of FBS, the growth of the DU-145 cells was increased by the coculture with both PrSCs compared to the growth of DU-145 cells only. As expected, high concentrations of FBS diminished the growth-stimulating effect of coculture with the PrSCs. Secretion of IGF-I into the conditioned medium by both PrSCs was also confirmed (Figure 1b). The PrSCs showed a mixture of fibroblasts expressing vimentin without SMA and myofibroblasts expressing both vimentin and SMA (Figure 1c). The addition of TGF-β1 significantly increased the proportion of myofibroblasts about two times in both PrSCs (Figure 1c). Theses two PrSCs were used for the following experiments.

Effects of TGF-β1 on expression of IGF axis components in PrSC. As shown in Figure 2, we found that TGF-β1 significantly increased the mRNA expression of IGF-I and IGFBP-3 in both PrSCs without an increase of other molecules of the IGF axis. Corresponding to the results in Figure 1, the expression of SMA was also increased by the treatment of TGF-β1. The amounts of IGF-I and IGFBP-3 in the conditioned medium of the TGF-β1-treated PrSC were also dtermined. Figure 3a shows that the amounts of IGFBP-3 were significantly increased by the treatment of TGF-β1 in both PrSCs corresponding to the results in Figure 2. In contrast, the amounts of IGF-I secreted from the PrSCs were decreased by TGF-β1 (Figure 3a). Because the anti-IGF-I antibody used in this assay preferentially detected free IGF-I unbound to IGFBP-3, the increased IGFBP-3 could have bound to the IGF-I, and prevented the IGF-I from being detected in this assay. To explore this possibility, immunoprecipitation assays were performed with the conditioned medium using anti-IGF-I and anti-IGFBP-3 antibodies. When the conditioned medium of PrSC was immunoprecipitated with anti-IGF-I antibody, Western blotting (Figure 3b) as well as ELISA assay (Figure 3a) showed that the amounts of IGF-I were decreased by the treatment with TGF-β1. However, when the immunoprecipitation was performed using the anti-IGFBP-3 antibody, in addition to the anti-IGF-I antibody, there was an apparent increase in the amounts of IGF-I in the conditioned medium by the treatment with TGF-β1 (Figure 3b). Theses results indicated that TGF-β1 increased the secretion of both IGF-I and IGFBP-3 by the PrSC. However, due to the increased IGFBP-3 production, the free IGF-I unbound to IGFBP-3 was conversely decreased.

The effect of TGF-β1 on the expression of the IGF axis components in the DU-145 cells was also examined. As shown in Figure 2, the DU-145 cells expressed IGF-IR, IGF-IIR, IGFBP-3, and IGFBP-6. The expressions of other molecules including IGF-I and SMA were under detectable levels (data not shown). While TGF-β1 increased the mRNA expression of IGFBP-3 in the DU-145 cells, it did not alter the expressions of any other molecules. Although TGF-β1 certainly increased the amounts of IGFBP-3 in the conditioned medium of DU-145 cells, the secreted levels were far less than that in the PrSCs (Figure 3).

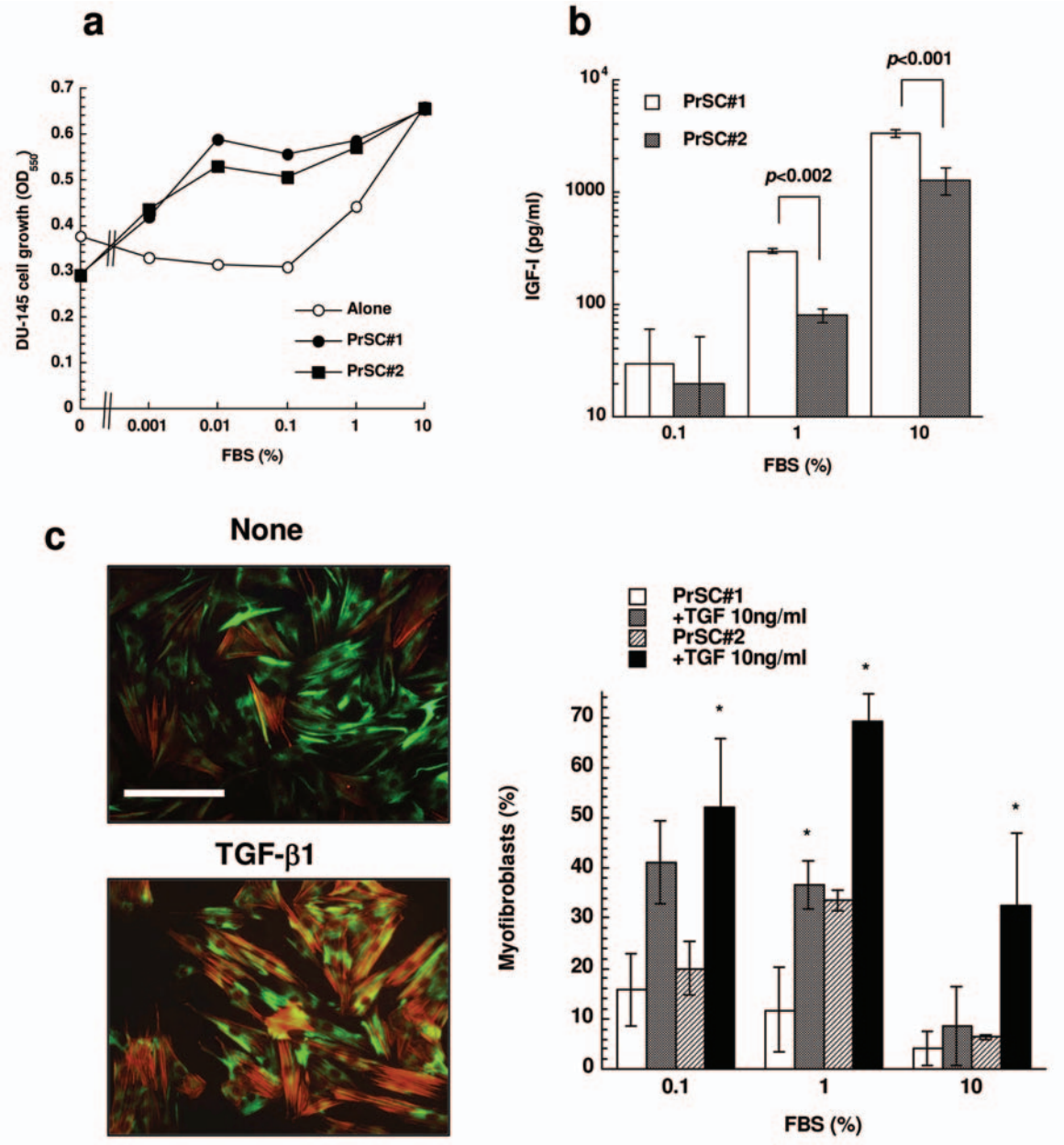

Figure 1. *Characteristics of PrSCs. (a) DU-145 cells were cultured alone (○) or cocultured with PrSC#1 (●) or PrSC#2 (■) with varying concentrations of FBS for 3 days. The growth of DU-145 was determined using rhodanile blue dye. Values are means of duplicate determinations (SE was less than 10%). (b) PrSCs were cultured with varying concentrations of FBS for 2 days and the amounts of IGF-I in the culture supernatants were determined. Values are mean±SD for triplicate determinations. (c) PrSCs were cultured with varying concentrations of FBS with or without 10 ng/ml TGF-β1 for 2 days. The percentage of myofibroblasts was evaluated by immunofluorecence using anti-vimentin (green) and anti-SMA (red). Values are mean±SD of triplicate determinations. *p<0.05 versus the values without TGF-β1. Photos are representative immunofluorescence results (bar=200 μm).*

Expression of IGF-I, IGFBP-3, and TGF-β1 in prostate cancer. The tissue arrays of human prostate cancer derived from 49 cases were immunostained with anti-IGF-I, anti-IGFBP-3, anti-TGF-β1, and anti-SMA antibodies (Figure 4 and Table I). The intensities of the immunostaining were scored separately in the epithelium and the stroma. Because the expression of SMA was restricted to the stroma, the postivie staining for SMA was used to discriminate between the epithelium and the stroma. IGF-I was highly expressed in the epithelium and in the stroma, staining immunohistochemically positive for 34 cases (69.4%) and 35 cases (71.4%), respectively. IGFBP-3 was similarly

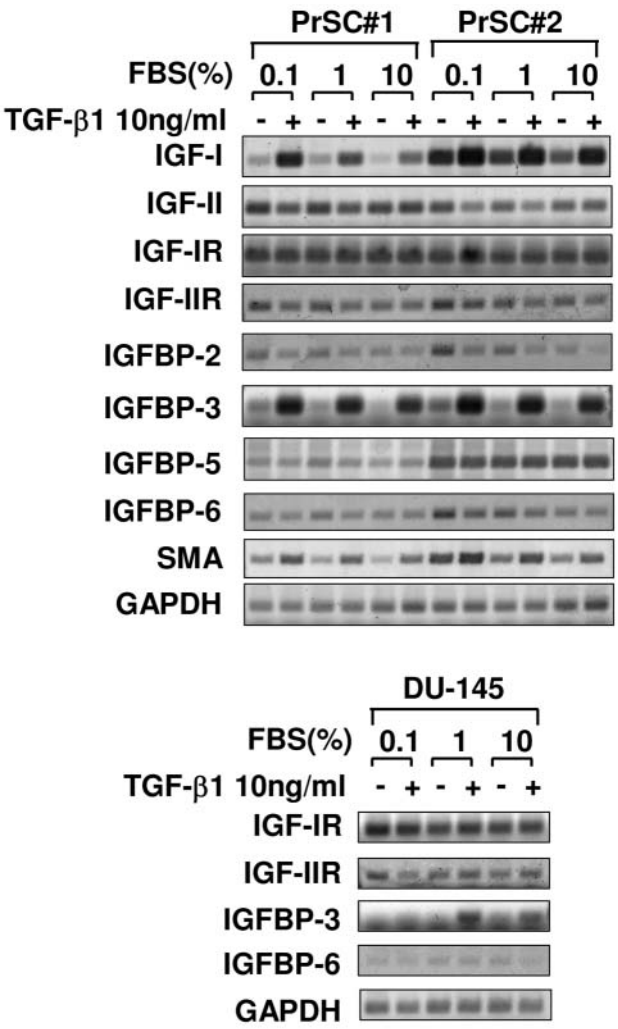

Figure 2. *Effect of TGF-β1 on expression of IGF axis components in PrSCs and DU-145 cells. PrSCs or DU-145 cells were cultured with varying concentrations of FBS with or without 10 ng/ml TGF-β1 for 2 days. Total RNAs were collected and RT-PCR of the indicated molecules was determined using specific primers.*

expressed in the epithelium (31 cases; 63.3%) and stroma (43 cases; 87.8%). The expression of TGF-β1 was also frequently observed in the epithelium (33 cases; 67.3%) and stroma (34 cases; 69.4%). Spearman's rank correlation analysis showed strong positive correlations of IGF-I expression in the stroma with TGF-β1 expression in the stroma ($r=0.551$, $p=0.0001$) and IGF-I expression in the stroma with IGFBP-3 expression in the stroma ($f=0.605$, $p<0.0001$). IGFBP-3 expression in the stroma also showed a strong positive correlation with TGF-β1 expression in the stroma ($r=0.547$, $p=0.0002$). Thus, these results correlated well with our *in vitro* results. The expressions of these molecules in the epithelium showed similar results. There were strong positive correlations of IGF-I with TGF-β1 ($r=0.690$, $p<0.0001$), IGF-I with IGFBP-3 ($r=0.722$, $p<0.0001$), and IGFBP-3 with TGF-β1 ($r=0.687$, $p<0.0001$) in the epithelium.

Restoration of IGF-I action by degradation of IGFBP-3. To determine the proteinase actions of prostate specific antigen (PSA) and MMP-7 on IGFBP-3 in our assay, their actions were first evaluated using NIH3T3 cells overexpressing IGF-IR as an independent model. As expected, PSA and MMP-7 degraded IGFBP-3, even in the presence of IGF-I (Figure 5a). Additionally, IGF-I transduced its mitogenic signal into IGF-IR-overexpressing cells and increased the phosphorylation of IGF-IR at the tyrosine residues, but IGFBP-3 inhibited the mitogenic action of IGF-I and decreased the tyrosine phosphorylation of IGF-IR (Figure 5b). However, PSA and MMP-7 restored the IGF-IR phosphorylation even in the presence of IGFBP-3 (Figure 5b).

To determine if these results occurred in the coculture system, the effect of TGF-β1 on the coculture in the presence of PSA or MMP-7 was examined. Since PrSC#1 secreted significantly more IGF-I than PrSC#2 (Figure 1), the PrSC#1 were used in this experiment. When TGF-β1 was added to the coculture of DU-145 cells and PrSC, TGF-β1 actually suppressed the growth of the DU-145 cells more strongly than that observed in monoculture (Figure 5c). Furthermore, PSA significantly restored the growth of the DU-145 cells in the TGF-β1-treated coculture, and MMP-7 moderately restored it (Figure 5d). Thus, these results indicated that without degradation of IGFBP-3 the growth of DU-145 cells in coculture with PrSC was suppressed by TGF-β1.

Discussion

Both the PrSCs secreted IGF-I and increased the growth of DU-145 prostate cancer cells in coculture (Figure 1a and 1b). Thus, results similar to our previous findings (21) were obtained with different PrSCs.

The PrSCs used were mixtures of fibroblasts and myofibroblasts (Figure 1c). As reported by others (22, 23), TGF-β1 clearly stimulated the PrSC to differentiate into myofibroblasts (Figure 1c). The TGF-β1 also increased the mRNA expression of IGF-I and IGFBP-3, in addition to an increase in SMA expression without an effect on IGF-II or other molecules of the IGF axis (Figure 2). Additionally TGF-β1 increased the secretion of IGF-I and IGFBP-3 from the PrSC, but biologically active IGF-I unbound to IGFBP-3 was decreased by the excess amounts of IGFBP-3 produced. The DU-145 cells expressed IGFBP-3 but not IGF-I and TGF-β1 increased the expression of IGFBP-3 (Figures 2 and 3). However, the secreted amounts of IGFBP-3 from the DU-145 cells were far less than from the PrSC. Therefore, the IGFBP-3 secreted from DU-145 cells did not substantially contribute to the coculutre with PrSC. The basal expression

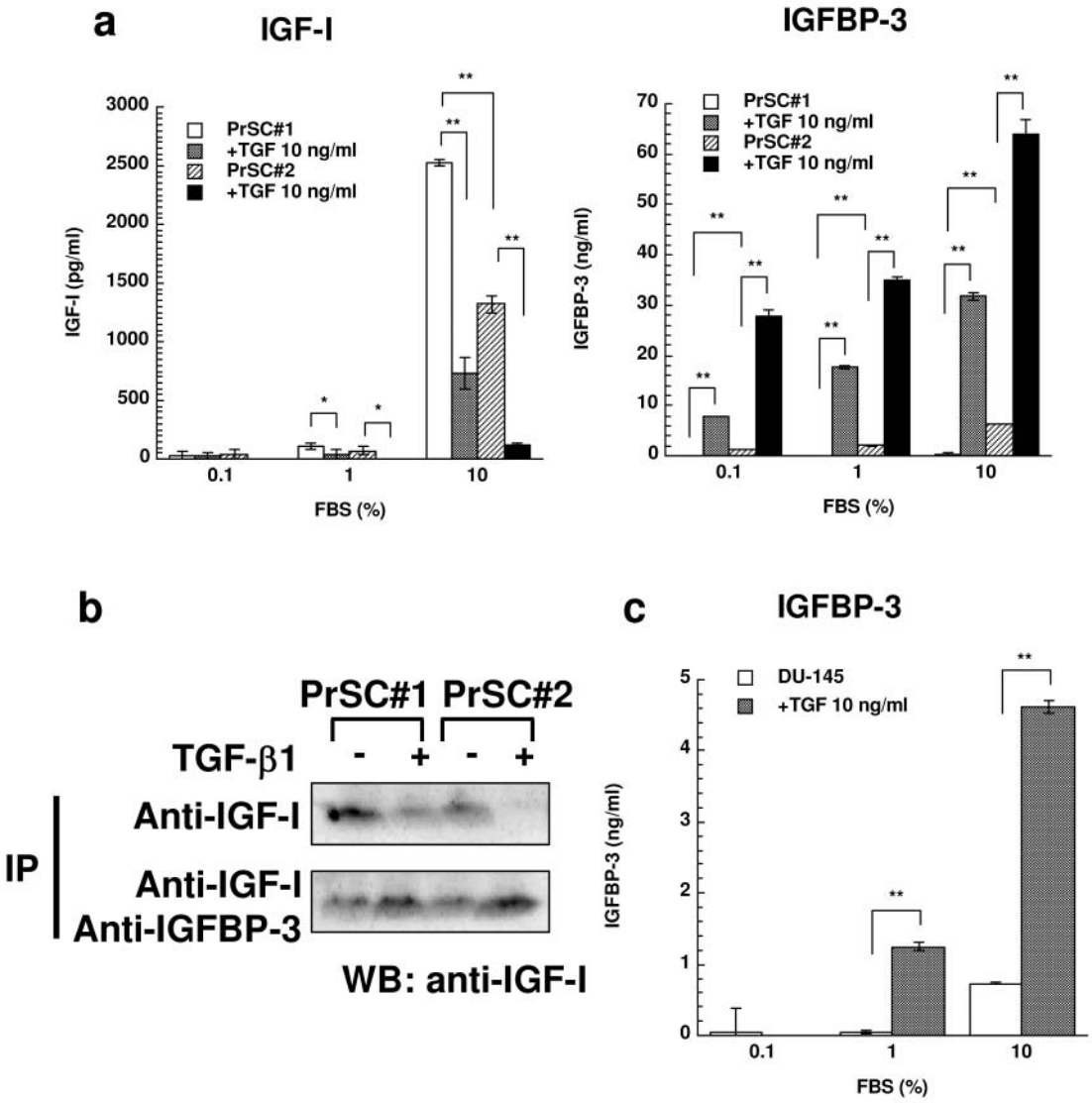

Figure 3. *Effect of TGF-β1 on IGF-I and IGFBP-3 production in PrSCs. (a) PrSCs were cultured with varying concentrations of FBS with or without 10 ng/ml TGF-β1 for 2 days. The amounts of IGF-I and IGFBP-3 in the culture supernatants were determined. Values are mean±SD of triplicate determinations. (b) PrSCs were cultured with 10% FBS with or without 10 ng/ml TGF-β1 for 2 days. The culture supernatant was immunoprecipitated with anti-IGF-I alone or both anti-IGF-I and anti-IGFBP-3 antibodies. IGF-I in the immunoprecipitates was immunoblotted with a biotinylated anti-IGF-I antibody. (c) DU-145 cells were cultured and the amounts of IGFBP-3 were determined as described in (a).*

level of IGF-I mRNA in PrSC#2 was higher than PrSC#1 (Figure 2), but the amounts of free IGF-I secreted by PrSC#2 were lower than PrSC#1 (Figure 1b). This was thought to be due to the excess amounts of IGFBP-3, as the basal level of IGFBP-3 secreted by PrSC#2 was higher than PrSC#1 (Figure 3). Several reports have shown that TGF-β1 and TGF-β2 increased the mRNA expression of IGF-I and the mRNA and protein expression of IGFBP-3 in human marrow stromal osteoblast precursor cells, human corneal fibroblasts and human intestinal fibroblasts (40-42). Thus, our results with the PrSCs were consistent with other reports and indicated that TGF-β1 action on stromal cells, such as fibroblasts, is universal. Furthermore, this suggested that both IGF-I and IGFBP-3 are transcriptionally regulated in the same way by the TGF-β1 signal.

IGF-I as well as TGF-β1 expression has been independently reported in both the epithelium and stroma of prostate cancer tissues (17, 43). Furthermore, TGF-β1 is highly expressed in the reactive stroma of prostate cancer (25, 29). Consistent with previous observations, IGF-I and TGF-β1 showed positive staining in both the epithelium and stroma of the prostate cancer tissue arrays used in the present

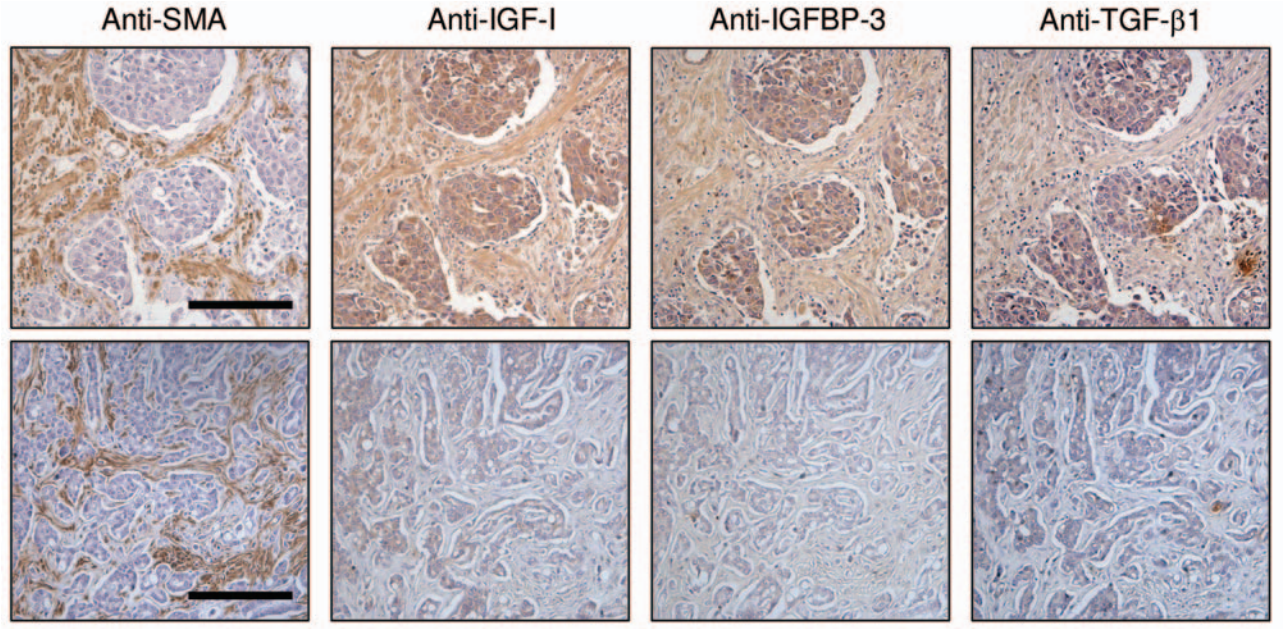

Figure 4. *Representative immunohistochemical staining of SMA, IGF-I, IGFBP-3, and TGF-β1 in human prostate cancer. Upper panels: positive staining; lower panels: negative staining (bar=200 μm).*

Table I. *Immunohistochemical expression of IGF-I, IGFBP-3, and TGF-β1 in human prostate cancer.*

		IGF-I (epithelium)				IGF-I (stroma)				IGFBP-3 (epithelium)				IGFBP-3 (stroma)				TGF-β1 (epithelium)			
Protein	n	–	+	++	Correlation*	–	+	++	Correlation*	–	+	++	Correlation*	–	+	++	Correlation*	–	+	++	Correlation*
n		15	17	17		14	18	17		18	14	17		6	26	17		16	17	16	
IGF-I (stroma)																					
–	14	9	3	2	r=0.470																
+	18	5	7	6	*p*=0.0011																
++	17	1	7	9																	
IGFBP-3 (epithelium)																					
–	18	11	6	1	r=0.690	9	5	4	r=0.305												
+	14	4	7	3	*p*<0.0001	2	7	5	*p*=0.0346												
++	17	0	4	13		3	6	8													
IGFBP-3 (stroma)																					
–	6	4	2	0	r=0.404	5	1	0	r=0.605	5	1	0	r=0.448								
+	26	9	9	8	*p*=0.0051	9	11	6	*p*<0.0001	10	9	7	*p*=0.0019								
++	17	2	6	9		0	6	11		3	4	10									
TGF-β1 (epithelium)																					
–	16	10	5	1	r=0.722	6	6	4	r=0.254	12	4	0	r=0.687	4	8	4	r=0.341				
+	17	5	10	2	*p*<0.0001	6	6	5	*p*=0.0781	5	7	5	*p*<0.0001	2	11	4	*p*=0.0182				
++	16	0	2	14		2	6	8		1	3	12		0	7	9					
TGF-β1 (stroma)																					
–	15	5	5	5	r=0.075	8	5	2	r=0.551	6	2	7	r=-0.009	4	10	1	r=0.547	6	4	5	r=0.137
+	24	8	8	8	*p*=0.6033	6	12	6	*p*=0.0001	8	11	5	*p*=0.9508	2	14	8	*p*=0.0002	8	10	6	*p*=0.3429
++	10	2	4	4		0	1	9		4	1	5		0	2	8		2	3	5	

The intesity of staining was scored as negative (–), weak positive (+), and strong positive (++). *Sperman's rank correlation.

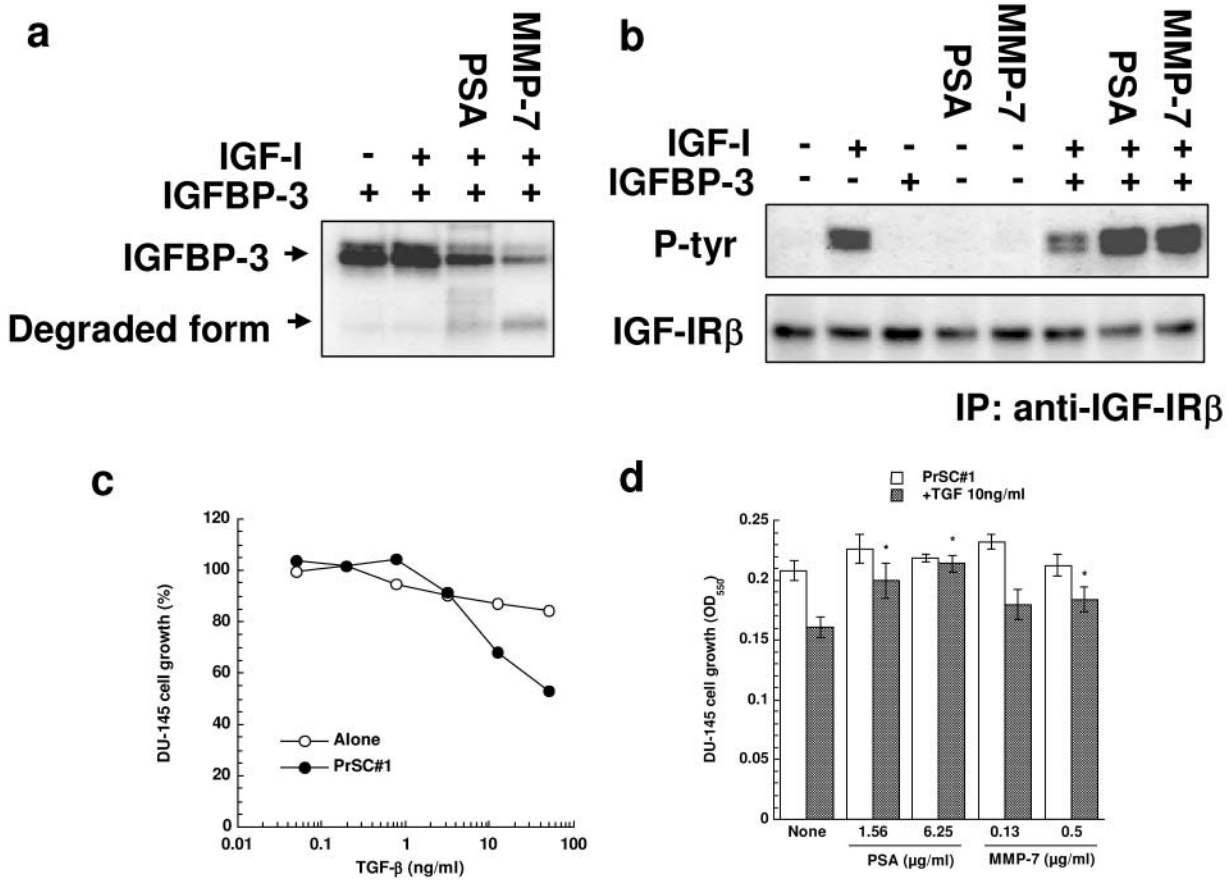

Figure 5. *Effect of PSA and MMP-7 on IGFBP-3 and IGF-I functions. (a) IGFBP-3 was incubated with PSA or MMP-7 in the presence of IGF-I. IGFBP-3 was immunoblotted by an anti-IGFBP-3 antibody. The degraded form of IGFBP-3 was also detected. (b) IGF-I, IGFBP-3, PSA, and MMP-7 were incubated in the indicated combinations. The reaction mixtures were added to IGF-IR overexpressing NIH3T3 cells. Cell lysates were immunoprecipitated with an anti-IGF-IRβ antibody and immunoblotted with anti-phosphotyrosine and anti-IGF-IRβ antibodies. (c) PrSC#1 were first cultured in medium with 1% FBS and varying concentrations of TGF-β1 for 2 days. DU-145 cells were cultured alone (○) or cocultured with the PrSC#1 (●) for 3 additional days. The growth of DU-145 cells was determined using rhodanile blue dye. The values are means of duplicate determinations. (SE was less than 10%.) (d) PrSC#1 were first cultured in medium with 1% FBS in the presence or absence of 10 ng/ml TGF-β1, and with or without the indicated concentrations of PSA or MMP-7 for 2 days. DU-145 cells were cocultured with PrSC#1 for 3 additional days. The growth of DU-145 was determined using rhodanile blue dye. Values are mean±SD of triplicate determinations. *$p<0.01$ versus the values without PSA and MMP-7.*

study (Figure 4 and Table I). Statistical analysis revealed significant positive correlations of IGF-I with TGF-β1, IGF-I with IGFBP-3, and IGFBP-3 with TGF-β1 expressions in the stroma (Table I). Thus, the immunohistochemical results correlated well with our *in vitro* results.

IGFBP-3 binds to IGF-I and inhibits the mitogenic action of IGF-I (12, 44). However, the degradation of IGFBP-3 by IGFBP-3 proteinases restores the mitogenic action of IGF-I (12, 44). PSA and MMP-7 have been reported to act as IGFBP-3 proteinases (39, 45). Our results confirmed that PSA and MMP-7 degraded IGFBP-3 and restored the mitogenic action of IGF-I, even in the presence of IGFBP-3 (Figure 5). DU-145 cells respond to IGF-I and their growth is increased by coculture with PrSC, but they do not express PSA and MMP-7 (21, 46). As expected, TGF-β1 actually suppressed the growth of the DU-145 cells in coculture with PrSC (Figure 5c), and that PSA and MMP-7 restored the growth of DU-145 cells in TGF-β1-treated coculture (Figure 5d). Thus, without degradation of IGFBP-3, the growth of prostate cancer cells in coculture with PrSC was suppressed by TGF-β1. However, since many prostate carcinomas secrete PSA and MMP-7 (47), they could efficiently receive the mitogenic signal of IGF-I secreted by TGF-β1-stimulated PrSC through degradation of IGFBP-3.

Singh *et al.* reported that TGF-β1-treated prostate stromal cells significantly decreased the rate of cell death of androgen-dependent human prostate cancer LNCaP cells in coculture (48) strongly supporting our current results. LNCaP cells express PSA, and respond to the mitogenic actions of IGF-I (16). It is thought that through the

degradation of IGFBP-3 by secreted PSA, LNCaP cells more efficiently utilize IGF-I secreted by TGF-β1-stimulated prostate stromal cells.

TGF-β1 actions on the growth of prostate cancer depend upon the balance between IGF-I and IGFBP-3 expression. Because TGF-β1 enhances the production of IGF-I by PrSC, it has a potential tumor stimulating activity. However, TGF-β1 also increases the production of IGFBP-3, a negative regulator of IGF-I action, and the excess IGFBP-3 effectively sequesters the mitogenic activity of IGF-I. Thus, this complex behavior of TGF-β1 on the IGF axis is one explanation for the pleiotropic activities of TGF-β1 on the growth of prostate cancer.

Acknowledgements

The authors thank Ms. K. Adachi and Ms. E. Satoh for their technical assistance.

References

1 Dvorak HF: Tumors: wounds that do not heal. N Eng J Med *315*: 1650-1659, 1986.
2 Tuxhorn JA, Ayala GE and Rowley DR: Reactive stroma in prostate cancer progression. J Urol *166*: 2472-2483, 2001.
3 Wernert N: The multiple roles of tumor stroma. Virchows Arch *430*: 433-443, 1997.
4 Picard O, Rolland Y and Poupon MF: Fibroblast-dependent tumorigenicity of cells in nude mice: implication for implantation of metastases. Cancer Res *46*: 3290-3294, 1986.
5 Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhau HE, Eschenbach AC and Chung LW: Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. Proc Natl Acad Sci USA *87*: 75-79, 1990.
6 Wiseman BS and Werb Z: Stromal effects on mammary gland development and breast cancer. Science *296*: 1046-1049, 2002.
7 Grossfeld GD, Hayward SW, Tlsty TD and Cunha GR: The role of stroma in prostatic carcinogenesis. Endocr Relat Cancer *5*: 253-270, 1998.
8 Micke P and Ostman A: Tumor-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer *45*: S163-S175, 2004.
9 Pollak M, Beamer W and Zhang JC: Insulin-like growth factors and prostate cancer. Cancer Metastasis Rev *17*: 383-390, 1999.
10 Delinassios JG: Cytocidal effect of human fibroblasts on HeLa cells *in vitro*. Biol Cell *59*: 69-77, 1987.
11 Jerome L, Shiry L and Leyland-Jones B: Deregulation of the IGF axis in cancer: epidemiological evidence and potential therapeutic interventions. Endocr Relat Cancer *10*: 561-578, 2003.
12 Hwa V, Oh Y and Rosenfeld RG: The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev *20*: 761-787, 1999.
13 Adam L, Crepin M, Lelong JC, Spanakis E and Israel L: Selective interactions between mammary epithelial cells and fibroblasts in co-culture. Int J Cancer *59*: 262-268, 1994.
14 Fürstenberger G and Senn HJ: Insulin-like growth factors and cancer. Lancet Oncol *3*: 298-302, 2002.
15 Marelli MM, Moretti RM, Dondi D, Motta M and Limonta P: Luteinizing hormone-releasing hormone agonists interfere with the mitogenic activity of the insulin-like growth factor system in androgen-independent prostate cancer cells. Endocrinology *140*: 329-334, 1999.
16 Iwamura M, Sluss PM, Casamento JB and Cockett AT: Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. Prostate *22*: 243-252, 1993.
17 Cardillo MR, Monti S, Di Silverio F, Gentile V, Sciarra F and Toscano V: Insulin-like growth factor (IGF)-I, IGF-II and IGF type I receptor (IGFR-I) expression in prostatic cancer. Anticancer Res *23*: 3825-3835, 2003.
18 Grzmil M, Hemmerlein B, Thelen P, Schweyer S and Burfeind P: Blockade of the type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. J Pathol *202*: 50-59, 2004.
19 Stattin P, Bylund A, Rinaldi S, Biessy C, Déchaud H, Stenman UH, Egevad L, Riboli E, Hallmans G and Kaaks R: Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. J Natl Cancer Inst *92*: 1910-1917, 2000.
20 Kawada M, Yoshimoto Y, Minamiguchi K, Kumagai H, Someno T, Masuda T, Ishizuka M and Ikeda D: A microplate assay for selective measurement of growth of epithelial tumor cells in direct coculture with stromal cells. Anticancer Res *24*: 1561-1568, 2004.
21 Kawada M, Inoue H, Masuda T and Ikeda D: Insulin-like growth factor I secreted from prostate stromal cells mediates tumor-stromal cell interactions of prostate cancer. Cancer Res *66*: 4419-4425, 2006.
22 Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD and Rowley DR: Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clin Cancer Res *8*: 2912-2923, 2002.
23 Peehl DM and Sellers RG: Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells. Exp Cell Res *232*: 208-215, 1997.
24 Ronnov-Jessen L and Petersen OW: Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. Lab Invest *68*: 696-707, 1993.
25 Tuxhorn JA, McAlhany SJ, Yang F, Dang TD and Rowley DR: Inhibition of transforming growth factor-β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. Cancer Res *62*: 6021-6025, 2002.
26 Steiner MS and Barrack ER: Transforming growth factor-β 1 overproduction in prostate cancer: effects on growth in vivo and *in vitro*. Mol Endocrinol *6*: 15-25, 1992.
27 Chang HL, Gillett N, Figari I, Lopez AR, Palladino MA and Derynck R: Increased transforming growth factor β expression inhibits cell proliferation *in vitro*, yet increases tumorigenicity and tumor growth of Meth A sarcoma cells. Cancer Res *53*: 4391-4398, 1993.
28 Cui W, Fowlis DJ, Bryson S, Duffie E, Ireland H, Balmain A and Akhurst RJ: TGF-β1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell *86*: 531-542, 1996.

29 San Francisco IF, DeWolf WC, Peehl DM and Olumi AF: Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. Int J Cancer *112*: 213-218, 2004.

30 Eastham JA, Truong LD, Rogers E, Kattan M, Flanders KC, Scardino PT and Thompson TC: Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. Lab Invest *73*: 628-635, 1995.

31 Barrett-Lee P, Travers M, Luqmani Y and Coombes RC: Transcripts for transforming growth factors in human breast cancer: clinical correlates. Br J Cancer *61*: 612-617, 1990.

32 Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG and Moses HL: TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science *303*: 848-851, 2004.

33 Kundu SD, Kim IY, Yang T, Doglio L, Lang S, Zhang X, Buttyan R, Kim SJ, Chang J, Cai X, Wang Z and Lee C: Absence of proximal duct apoptosis in the ventral prostate of transgenic mice carrying the C3(1)-TGF-β type II dominant negative receptor. Prostate *43*: 118-124, 2000.

34 Kimura G, Kasuya J, Giannini S, Honda Y, Mohan S, Kawachi MH, Akimoto M and Fujita-Yamaguchi Y: Insulin-like growth factor (IGF) system components in human prostatic cancer cell-lines: LNCaP, DU145, and PC-3 cells. Int J Urol *3*: 39-46, 1996.

35 Pekonen F, Nyman T, Ilvesmäki V and Partanen S: Insulin-like growth factor binding proteins in human breast cancer tissue. Cancer Res *52*: 5204-5207, 1992.

36 Rutanen E, Nyman T, Lehtovirta P, Ammälä M and Pekonen F: Suppressed expression of insulin-like growth factor binding protein-1 mRNA in the endometrium: a molecular mechanism associating endometrial cancer with its risk factors. Int J Cancer *59*: 307-312, 1994.

37 Yang D, Howard OM, Chen Q and Oppenheim JJ: Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-β1 express functional C-C chemokine receptor 6. J Immunol *163*: 1734-1741, 1999.

38 Kawada M, Masuda T, Ishizuka M and Takeuchi T: 15-Deoxyspergualin inhibits Akt kinase activation and phosphatidylcholine synthesis. J Biol Chem *277*: 27765-27771, 2002.

39 Miyamoto S, Yano K, Sugimoto S, Ishii G, Hasebe T, Endoh Y, Kodama K, Goya M, Chiba T and Ochiai A: Matrix metalloproteinase-7 facilitates insulin-like growth factor bioavailability through its proteinase activity on insulin-like growth factor binding protein 3. Cancer Res *64*: 665-671, 2004.

40 Kveiborg M, Flyvbjerg A, Eriksen EF and Kassem M: Transforming growth factor-β1 stimulates the production of insulin-like growth factor-I and insulin-like growth factor-binding protein-3 in human bone marrow stromal osteoblast progenitors. J Endrocrinol *169*: 549-561, 2001.

41 Izumi K, Kurosaka D, Iwata T, Oguchi Y, Tanaka Y, Mashima Y and Tsubota K: Involvement of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in corneal fibroblasts during corneal wound healing. Invest Ophthalmol Vis Sci *47*: 591-598, 2006.

42 Simmons JG, Pucilowska JB, Keku TO and Lund PK: IGF-I and TGF-β1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. Am J Physiol Gastrointest Liver Physiol *283*: G809-G818, 2002.

43 Gerdes MJ, Larsen M, McBride L, Dang TD, Lu B and Rowley DR: Localization of transforming growth factor-β1 and type II receptor in developing normal human prostate and carcinoma tissues. J Histochem Cytochem 46: 379-388, 1998.

44 Peehl DM, Cohen P and Rosenfeld RG: The insulin-like growth factor system in the prostate. World J Urol *13*: 306-311, 1995.

45 Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC and Rosenfeld RG: Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab *75*: 1046-1053, 1992.

46 Powell WC, Knox JD, Navre M, Grogan TM, Kittelson J, Nagle RB and Bowden GT: Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res *53*: 417-422, 1993.

47 Hashimoto K, Kihira Y, Matuo Y and Usui T: Expression of matrix metalloproteinase-7 and tissue inhibitor of metallo-proteinase-1 in human prostate. J Urol *160*: 1872-1876, 1998.

48 Singh H, Dang TD, Ayala GE and Rowley DR: Transforming growth factor-β1 induced myofibroblasts regulate LNCaP cell death. J Urol *172*: 2421-2425, 2004.

Received November 28, 2007
Revised January 21, 2008
Accepted February 4, 2008