Insulin-like Growth Factor I Secreted from Prostate Stromal Cells Mediates Tumor-Stromal Cell Interactions of Prostate Cancer

Manabu Kawada, Hiroyuki Inoue, Tohru Masuda, and Daishiro Ikeda

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

Abstract

Prostate cancer shows high expression of type I insulin-like growth factor (IGF-I) receptor (IGF-IR) and prostate stromal cells (PrSC) produce IGF-I. Although high plasma level of IGF-I is a risk factor of prostate cancer, the significance of the prostate stromal IGF-I in the regulation of prostate cancer remains elusive. Here we show that the stromal IGF-I certainly regulates the development of prostate cancer. Coinoculation of PrSC increased the growth of human prostate cancer LNCaP and DU-145 tumors in severe combined immunodeficient mice. The conditioned medium of PrSC, as well as IGF-I, induced phosphorylation of IGF-IR and increased the growth of LNCaP and DU-145 cells. PrSC, but not LNCaP and DU-145 cells, secreted significant amounts of IGF-I. Coculture with PrSC increased the growth of DU-145 cells *in vitro* but the pretreatment of PrSC with small interfering RNA of IGF-I did not enhance it. Furthermore, various chemical inhibitors consisting of 79 compounds with ∼60 different targets led to the finding that only IGF-IR inhibitor suppressed the PrSC-induced growth enhancement of DU-145 cells. Thus, these results show that the prostate stromal IGF-I mediates tumor-stromal cell interactions of prostate cancer to accelerate tumor growth, supporting the idea that the IGF-I signaling is a valuable target for the treatment of prostate cancer. (Cancer Res 2006; 66(8): 4419-25)

Introduction

The insulin-like growth factor (IGF) axis is involved in the regulation of cellular proliferation, differentiation, and apoptosis (1, 2). The IGF axis consists of two major ligands (IGF-I and IGF-II), two cell-surface receptors (IGF-IR and IGF-IIR), 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 (2, 3). IGF-I binds to IGF-IR and the tyrosine kinase of the cytoplasmic domain of IGF-IR transduces the IGF-I signaling into cells (1, 2).

Various lines of evidence suggest the undoubted involvement of the IGF axis in the development of prostate cancer (4). Prostate cancer cells respond to mitogenic actions of IGF-I (5, 6) and prostate cancer tissues show high expression of IGF-IR (7, 8). Androgen independence is a major obstacle to the treatment of prostate cancer. Several reports have shown that the androgen-independent prostate cancer cells increase the expressions of IGF-I and IGF-IR compared with the androgen-dependent cancer cells (9, 10). Anti-IGF-IR antibodies, IGF-IR inhibitors, and antisense oligonucleotides to IGF-IR successfully suppress prostate cancer cell growth *in vitro* and *in vivo* (11–13). Thus, IGF-I signaling is one of the valuable targets for the treatment of prostate cancer.

A number of recent epidemiologic studies have suggested that reduced plasma levels of IGFBP-3, increased plasma levels of IGF-I, and an increased ratio of IGF-I to IGFBP-3 are associated with an increased risk for the development of several common cancers, particularly those of the breast, prostate, lung, and colon cancers (1, 2, 14). Although prostate stroma secretes IGF-I (7), the significance of the stromal IGF-I in the regulation of prostate cancer growth remains elusive.

Solid tumors are composed of tumor cells and surrounding stroma including extracellular matrix, fibroblasts, macrophages, and endothelial cells (15, 16). The growth of tumor cells as well as that of normal epithelial cells is regulated by the stromal cells through diffusible factors and their adhesion (17, 18). Thus, the stromal cells not only accelerate the growth of tumor cells (19, 20) but also suppress it (21, 22) *in vitro* and *in vivo*. Tumor-stromal cell interactions significantly contribute to the development of some cancers such as the breast and prostate cancers (23, 24). We have recently established the *in vitro* coculture system of prostate cancer cells and prostate stromal cells (PrSC; ref. 25). Using our coculture system, we have found that PrSC increased the growth of human prostate cancer LNCaP and DU-145 cells (25).

In this study, we have investigated the significance of the prostate stromal IGF-I in the regulation of prostate cancer growth. Our results indicate that the local secretion of IGF-I in the prostate stroma plays a critical role in the development of prostate cancer as well as the circulating IGF-I. Thus, this study promotes the idea that the IGF-I signaling is a valuable target for the treatment of prostate cancer.

Materials and Methods

Reagents. Rhodanile blue was purchased from Aldrich (Milwaukee, WI). Insulin, hydrocortisone, and 5α-dihydrotestosterone were obtained from Sigma (St. Louis, MO). Transferrin was from Wako Pure Chemical Industries (Tokyo, Japan). Recombinant human basic fibroblast growth factor (bFGF) was from Pepro Tech (London, United Kingdom). Recombinant human IGF-I and IGF-II were from R&D Systems (Minneapolis, MN). Human plasma fibronectin was from Chemicon (Temecula, CA). Antibodies used were antihuman fibronectin (A 0245; Dako, Glostrup, Denmark); anti-IGF-IRβ and anti-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-tyrosine (05-321; Upstate Biotechnology, Lake Placid, NY); and anti–phosphospecific IRS-1 (PC463; Oncogene, La Jolla, CA). The SCADS inhibitor kit I consisting of 79 chemical inhibitors with ∼60 different targets was kindly provided by Screening Committee of Anticancer Drugs (Japan).

Cells. Human prostate cancer LNCaP cells were obtained from Dainippon Seiyaku (Osaka, Japan). Human prostate cancer DU-145 cells and human normal PrSC were from American Type Culture Collection

(Manassas, VA) and BioWhittaker (Walkersville, MD), respectively. Prostate cancer cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH), 100 units/mL penicillin G, and 100 µg/mL streptomycin at 37°C with 5% CO_2. PrSC were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin G and 100 µg/mL streptomycin, ITH (5 µg/mL insulin, 5 µg/mL transferrin, and 1.4 µmol/L hydrocortisone), and 5 ng/mL bFGF at 37°C with 5% CO_2.

Tumor formation in severe combined immunodeficient mice. Male severe combined immunodeficient (SCID) mice, 6 weeks old, were purchased from Charles River Breeding Laboratories (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. The prostate tumor cells (8×10^6) were trypsinized and resuspended with or without PrSC (8×10^6) in 0.3 mL of 10% FBS-DMEM and then combined with 0.5 mL of growth factor-reduced Matrigel (BD Biosciences, Bedford, MA). One hundred microliters of the cell suspension (1×10^6 cells) were injected s.c. in the left lateral flank of mice. Five mice were used for each experimental set. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × $width^2$) / 2. After the indicated times, tumors were surgically dissected.

Preparation of conditioned medium. PrSC (2.5×10^4 cells) were plated in a 60-mm dish in DMEM supplemented with 0.1% FBS and ITH and cultured for 4 days. The cultured supernatants were collected and the 100-µL aliquots were added into 96-well plates. Then 10 µL of prostate cancer cells (5,000 cells) were inoculated into the well containing the conditioned medium and cultured for 4 days. Cell growth was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) as described (26).

Cell growth. Prostate cancer cells (5×10^3 per well) were inoculated into 96-well plates in DMEM supplemented with 0.1% FBS and ITH and cultured for 4 days. The growth was determined as described above. To precoat 96-well plates with fibronectin, 50 µL of fibronectin (50 µg in PBS) were added into a well and the plate was kept at 4°C overnight. The fibronectin-coated plate was washed with PBS and cells were inoculated as described above.

Preparation of cell lysates and Western blotting. Prostate cancer cells (3×10^5) were cultured in serum-free DMEM overnight and treated with IGF or PrSC conditioned medium for 10 minutes. The cell lysates were prepared and directly applied onto Western blotting or immunoprecipitated with antibodies as described (27). PrSC conditioned medium was prepared from PrSC cultured with 0.1% or 1% dialyzed FBS for 2 days.

Reverse transcription-PCR analysis. PrSC (10^6 cells) were inoculated into the lower chambers of Transwell plates (Costar, Cambridge, MA) and prostate cancer cells (5×10^5) were plated into the upper chambers. The cells were cultured in DMEM supplemented with 0.1% FBS and ITH for 1 or 2 days and the total RNAs were isolated using the RNeasy Minikit (Qiagen GmbH, Hilden, Germany). The cDNAs were synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) with the same quantity of RNA (1 µg) and amplified using Taq DNA polymerase (Promega). Specific primers used were as follows: IGFBP-3 (158 bp), 5′-CGCCAGCTCCAGGAAATG-3′ (sense) and 5′-GCTGTCTTTAG-CATGCCCTTTCT-3′ (antisense), IGF-I (342 bp; ref. 28), IGF-II (359 bp; ref. 28), IGF-IR (190 bp; ref. 28), IGF-IIR (370 bp; ref. 28), IGFBP-1 (445 bp; ref. 29), IGFBP-2 (497 bp; ref. 30), IGFBP-4 (594 bp; ref. 30), IGFBP-5 (370 bp; ref. 30), IGFBP-6 (524 bp; ref. 29), and glyceraldehyde-3-phosphate dehydrogenase (246 bp; ref. 31) were reported elsewhere. PCRs were optimized for each set of primers and done 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), and evaluated by an image analyzer FLA-5000 (Fujifilm, Tokyo, Japan).

Measurement of IGF-I. PrSC and prostate cancer cells were inoculated alone or coinoculated into 12-well plates in DMEM supplemented with ITH and the indicated concentrations of FBS. The cells were cultured for the indicated times and the supernatants of the cell cultures were withdrawn.
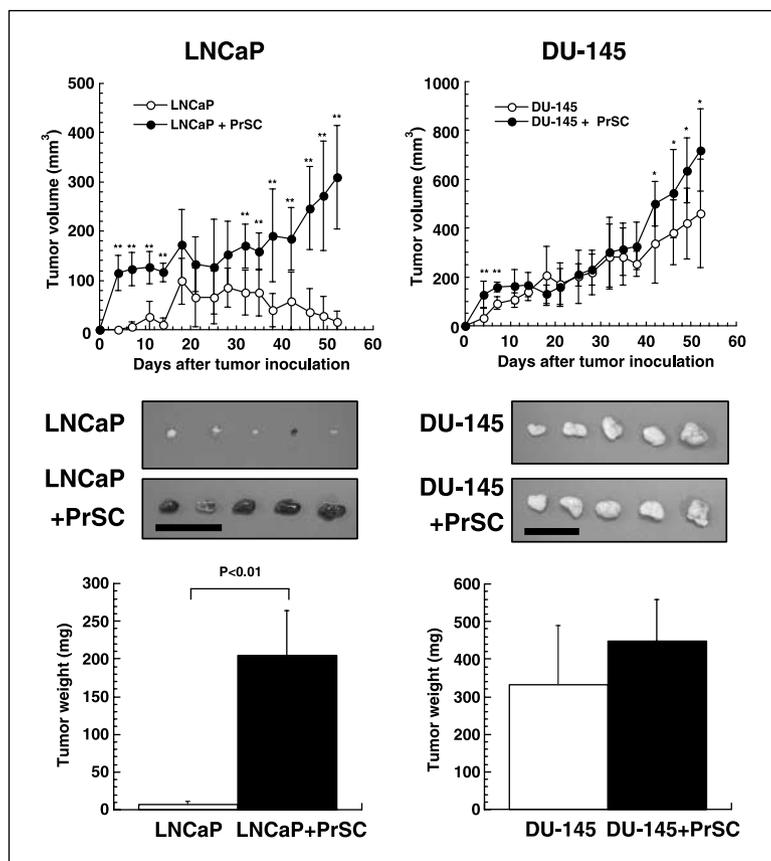
Figure 1. Effect of coinoculation of PrSC on growth of prostate tumors. LNCaP and DU-145 cells were inoculated s.c. with (●) or without (○) PrSC in SCID male mice. The tumor growth was measured (*top*) and the mice were sacrificed on day 52 for LNCaP and DU-145 tumors. The tumors were excised (*middle*; bar, 3 cm) and weighted (*bottom*). *Points* and *columns,* mean of five mice; *bars,* SD. **, $P < 0.01$; *, $P < 0.05$, versus the values without PrSC.

The amounts of IGF-I were determined using human IGF-I Quantikine (R&D Systems).

Coculture experiment. The coculture experiment using rhodanile blue dye was done as previously described (25). PrSC were first inoculated in 96-well plates at 5,000 per well in 100 μL of DMEM supplemented with 0.1% FBS and ITH. Test samples were added into the well and the cells were cultured for 2 days. Then 10 μL of prostate cancer cell suspension (5,000 cells) in serum-free DMEM were inoculated onto a monolayer of PrSC and the cells were further cultured for 3 days. For monoculture of prostate cancer cells, only assay medium with test samples was first incubated for 2 days at 37°C, and then prostate cancer cells were inoculated as described above and further cultured for 3 days.

Experiment of small interfering RNA. We designed Stealth small interfering RNA (siRNA; Invitrogen, Carlsbad, CA) of human IGF-I (5′-UGUCUCCACACACGAACUGAAGAGC-3′) and control (5′-UGUGACCUA-CACACGCAAUCAGAGC-3′). The duplex oligoribonucleotides were added into the culture of PrSC using Lipofectamine 2000 (Invitrogen) according to the method of the manufacturer. Coculture experiment and reverse transcription-PCR (RT-PCR) analysis were assessed as described above.

Statistical analysis. All data are representatives of two or three independent experiments with similar results. Statistical analysis was done by using Student's t test.

Results

Effect of coinoculation of PrSC on growth of prostate tumors. We have recently established the *in vitro* coculture system using rhodanile blue dye and found that PrSC increased the growth of human prostate cancer LNCaP and DU-145 cells by the coculture (25). To evaluate our previous finding *in vivo*, we examined the effect of PrSC on the tumor growth of LNCaP and DU-145 cells *in vivo*. Tuxhorn et al. (32) reported that LNCaP cells rarely form tumors in male SCID mice but PrSC enhance the tumor incidence and growth. As shown in Fig. 1, LNCaP cells formed only palpable tumors but the coinoculation of PrSC significantly enhanced the tumorigenicity and increased the LNCaP tumors in male SCID mice. Although DU-145 cells alone formed tumors, the coinoculation of PrSC also apparently increased the DU-145 tumors in SCID mice (Fig. 1). Thus, these results showed that PrSC increased both LNCaP and DU-145 tumors *in vivo*, indicating that these *in vivo* results were comparable to our previous finding of the coculture *in vitro* (25).

Modulation of prostate cancer cell growth by secreted factors from PrSC. We next examined whether the secreted factors from PrSC modulate tumor growth *in vitro*. As a result, the conditioned medium of PrSC also significantly increased the growth of LNCaP and DU-145 cells (Fig. 2A). Thus, this result correlates well with the *in vivo* results (Fig. 1), indicating that the secreted factors from PrSC certainly modulate the growth of prostate cancer cells. When LNCaP cells were cultured in the PrSC conditioned medium, LNCaP cells spread and adhered to the well (Fig. 2B) but the morphology of DU-145 cells was not affected (data not shown). Because fibronectin is one of major extracellular matrix secreted by stromal cells (17), we determined whether PrSC secretes fibronectin into the conditioned medium. Antifibronectin antibody detected the significant amounts of fibronectin in the PrSC conditioned medium but not in the control medium (Fig. 2C). Furthermore, antifibronectin antibody inhibited the adhesion of LNCaP cells to the well (Fig. 2B). When fibronectin was precoated on the well, only the growth of LNCaP cells was significantly increased (Fig. 2D). Thus, one of modulators of prostate tumor growth in the PrSC conditioned medium, especially for LNCaP cells, is fibronectin.
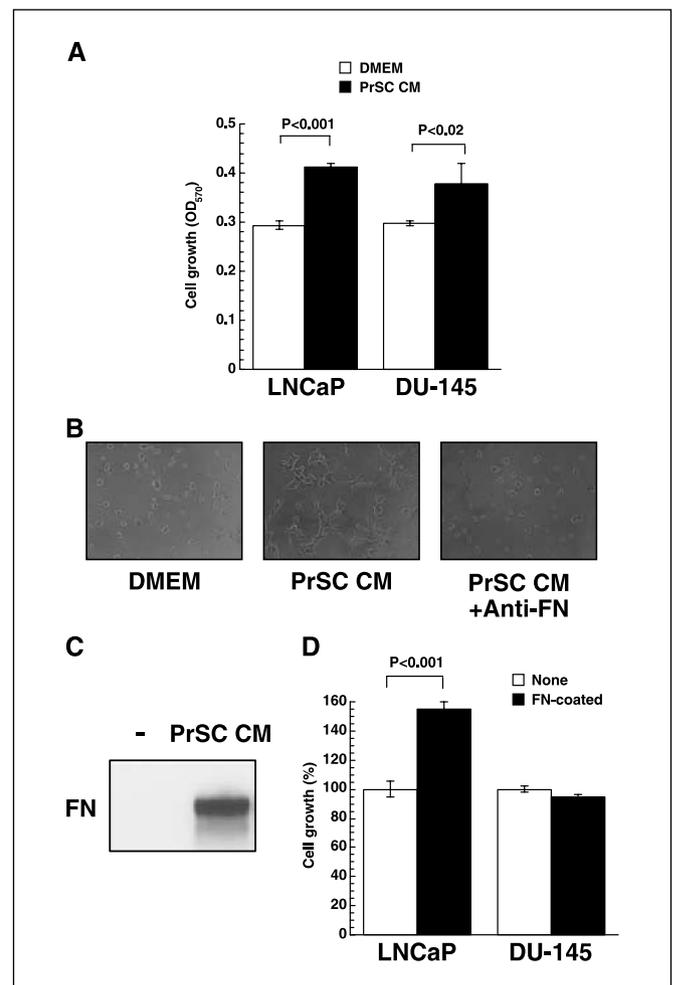
Figure 2. Effect of PrSC conditioned medium on prostate cancer cells. *A,* prostate cancer cells were cultured in the control medium (□) or the PrSC conditioned medium (■) that were prepared from PrSC cultured for 4 days, and the cell growth was determined using MTT. *Columns,* mean of triplicate determinations; *bars,* SD. *B,* morphology of LNCaP cells cultured in the PrSC conditioned medium. LNCaP cells were cultured in the control medium (DMEM) or the PrSC conditioned medium with or without 25 μL/mL antifibronectin (*anti-FN*) antibody for 1 hour. *C,* the control medium (DMEM; −) and the PrSC conditioned medium were applied onto Western blotting with antifibronectin antibody. *D,* prostate cancer cells were inoculated into the wells precoated with (■) or without fibronectin (□) and cultured for 4 days. The cell growth was determined using MTT. *Columns,* mean of triplicate determinations; *bars,* SD.

Effect of IGF-I on growth of prostate cancer cell lines. To investigate the involvement of IGF axis in our coculture system of prostate cancer cells and PrSC, we assessed the effect of IGF-I on the growth of prostate cancer cell lines *in vitro* compared with IGF-II. As shown in Fig. 3A, the growth of DU-145 cells was significantly increased by the addition of IGF-I. As reported by others (6, 13), the growth of LNCaP cells was also increased by IGF-I in the presence of 5α-dihydrotestosterone. On the other hand, IGF-II did not increase the growth of the two prostate cancer cell lines (Fig. 3A).

IGF-I activates IGF-IR tyrosine kinase, and the activated IGF-IR phosphorylates IRS-I, one of the major substrate of the IGF-IR (1, 2). As expected, IGF-I, but not IGF-II, increased the tyrosine phosphorylation of IGF-IR in LNCaP and DU-145 cells (Fig. 3B). Furthermore, IRS-1 was also significantly phosphorylated by IGF-I in DU-145 cells. We did not detect the phosphorylation of IRS-1 in
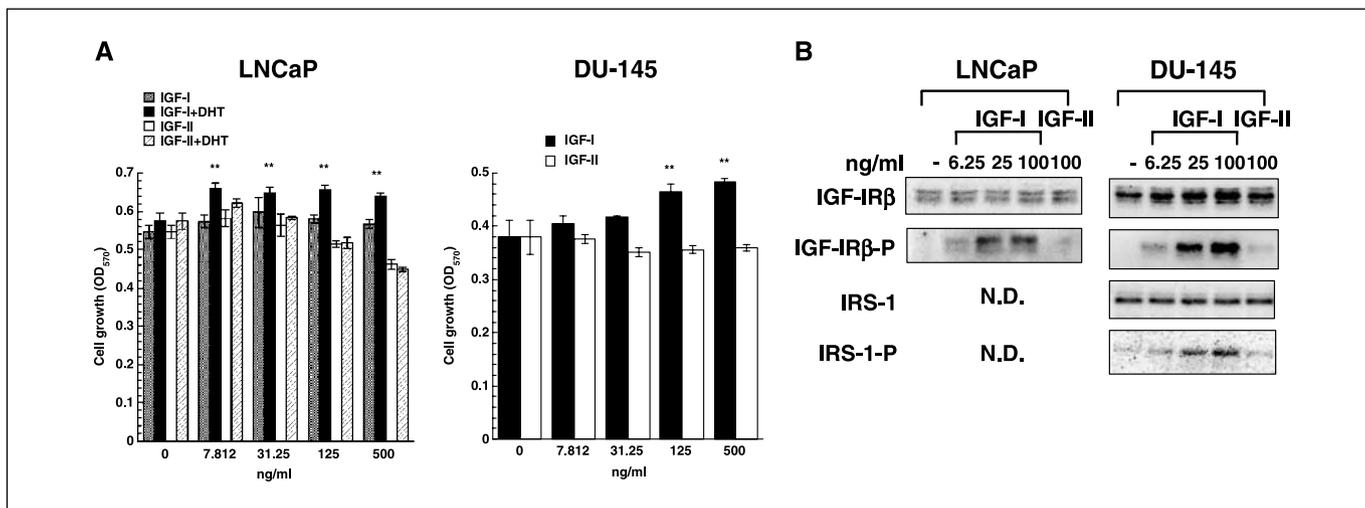
Figure 3. Effect of IGFs on prostate cancer cells. *A,* prostate cancer cells were cultured with the indicated concentrations of IGF-I or IGF-II for 4 days. For LNCaP cells, 1 nmol/L 5α-dihydrotestosterone was also added into the culture. The cell growth was determined using MTT. *Columns,* mean of triplicate determinations; *bars,* SD. **, $P < 0.001$, versus none. *B,* IGF signaling in prostate cancer cells. Cells were precultured in serum-free medium overnight and the indicated concentrations of IGF-I or IGF-II were added. After 10 minutes, the cell lysates were prepared and applied onto Western blotting. The indicated proteins were detected by the specific antibodies. For the detection of phosphorylated IGF-IRβ, the cell lysates were first immunoprecipitated with anti-IGF-IRβ before Western blotting with anti-phosphotyrosine antibody. *N.D.,* not detected.

LNCaP cells because of the deficient expression of IRS-I (33). Thus, IGF-I, but not IGF-II, transduced its signaling in both LNCaP and DU-145 cell lines, of which the growth was up-regulated by PrSC *in vitro* and *in vivo.*
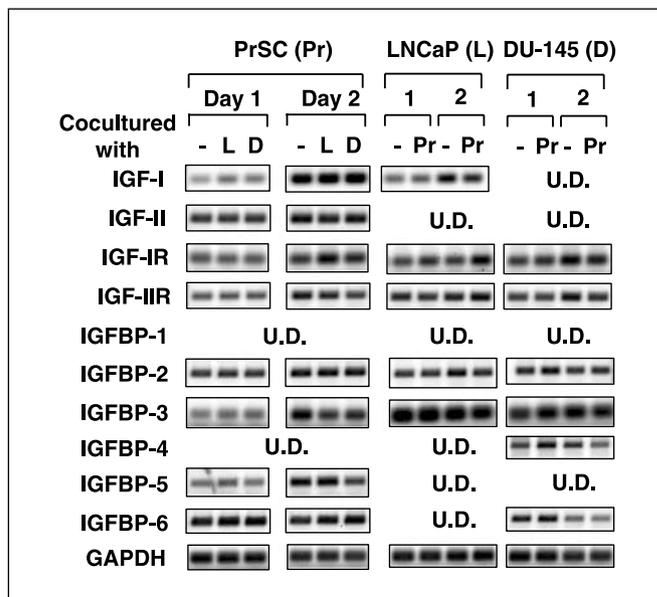
Expressions of IGF axis in prostate cancer cells and PrSC. We next examined the expressions of various molecules that consist of IGF axis. Prostate cancer cell lines and PrSC were cultured alone or cocultured with each other, and the mRNA expressions of IGF-I, IGF-II, IGF-IR, IGF-IIR, and six IGFBPs were determined. Almost all molecules investigated here were expressed in PrSC but some molecules were not detected in the two prostate cancer cell lines (Fig. 4). They expressed two IGF receptors, IGF-IR and IGF-IIR, and IGFBP-2 and IGFBP-3, but not IGF-II and IGFBP-5. Among them, only LNCaP cells expressed IGF-I (Fig. 4). Whereas the coculture of PrSC did not affect the expressions of IGF axis molecules in the two prostate cancer cell lines, the coculture of LNCaP or DU-145 cells resulted in a slight increase of IGF-I expression in PrSC at day 1.

Because LNCaP and DU-145 cells responded to the mitogenic action of IGF-I (Fig. 3), we determined the amounts of IGF-I secreted. As a result, PrSC certainly secreted the significant amounts of IGF-I into the conditioned medium (Fig. 5A). Although a slight amount of IGF-I was detected in LNCaP and DU-145 cells (Fig. 5B), the secreted amounts were very low compared with that of PrSC. Furthermore, the PrSC conditioned medium induced tyrosine phosphorylation of IGF-IR in DU-145 cells (Fig. 5D), indicating that IGF-I in the PrSC conditioned medium undoubtedly transduced the mitogenic signaling into the cancer cells. Whereas RT-PCR experiment (Fig. 4) suggested that the coculture of LNCaP or DU-145 cells could enhance the secretion of IGF-I from PrSC, IGF-I secretion was not increased by the coculture and it was decreased instead (Fig. 5C).

Growth suppression of DU-145 cells in coculture with PrSC by inhibition of IGF-I signaling. We ascertained whether the inhibition of IGF-I signaling results in the suppression of prostate cancer cell growth in the coculture with PrSC. As shown in Fig. 6A,

IGF-I siRNA successfully inhibited the expression of IGF-I, but not that of IGF-II, in PrSC. The growth of DU-145 cells was increased by the coculture with PrSC (Fig. 6B). However, when PrSC were first treated with the siRNA to inhibit the IGF-I production, the growth of DU-145 cells in the coculture was significantly suppressed (Fig. 6B). IGF-I siRNA treatment did not affect the growth of DU-145 cells in the monoculture. Thus, this result supports the idea that IGF-I secreted by PrSC modulates the growth of prostate

Figure 4. Expressions of IGF axis in prostate cancer cells and PrSC. PrSC (Pr) were inoculated into the lower chambers of Transwell plates and LNCaP (L) or DU-145 (D) cells were plated into the upper chambers. The plates were cultured for 1 or 2 days and the total RNAs were collected from each chamber. RT-PCR of the indicated molecules was assessed using the specific primers. —, medium without cocultured cells; *U.D.,* under detectable level.

Figure 5. IGF-I production in PrSC and prostate cancer cells. *A,* PrSC were cultured in the medium with the indicated concentrations of FBS and the amounts of IGF-I in the culture supernatant were determined. *B* and *C,* prostate cancer cells or PrSC were cultured alone (*B*) or PrSC were cocultured for 1 or 2 days with the indicated prostate cancer cells (*C*) in the medium with 10% FBS and the amounts of IGF-I in the culture supernatant were determined. *Columns,* mean of duplicate determinations. Each SE is <10%. *D,* DU-145 cells were precultured in serum-free medium overnight and the control medium (DMEM; −) or the PrSC conditioned medium (+) was added. After 10 minutes, the cell lysates were prepared and the indicated proteins were detected as described in Fig. 3*B*. The PrSC conditioned medium was prepared from PrSC cultured for 2 days with the indicated concentrations of dialyzed FBS.
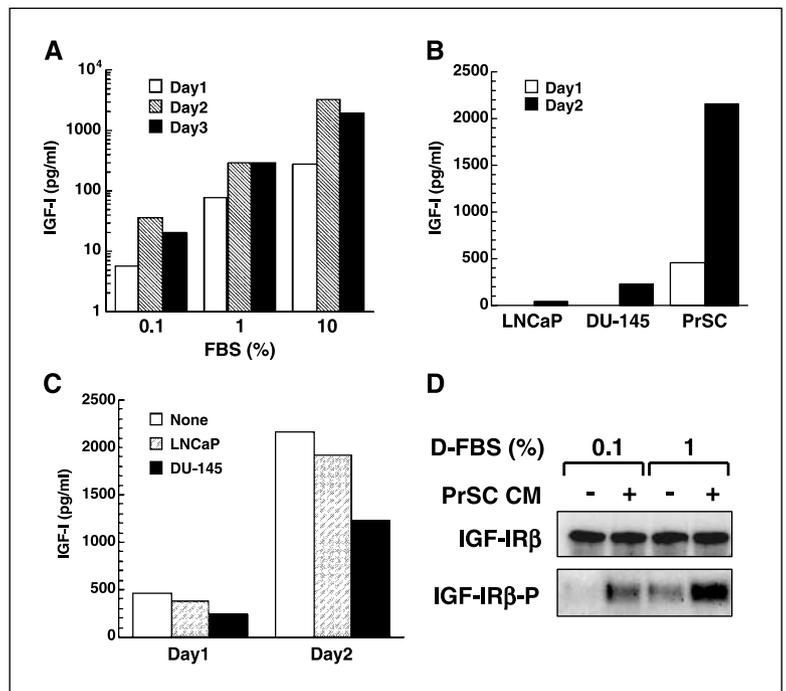
cancer cells by the coculture. To verify that IGF-I signaling is a target for tumor-stromal cell interactions of prostate cancer, we examined the effects of various chemical inhibitors on the coculture of DU-145 cells and PrSC using the SCADS inhibitor kit I consisting of 79 inhibitors with ∼60 different targets. As a result, only AG1024, a specific inhibitor of IGF-IR tyrosine kinase, exhibited the desired effect (Fig. 7*A*). As shown in Fig. 7*B*, AG538 (11), a less specific inhibitor of IGF-IR, inhibited the growth of DU-145 cells equally both in the monoculture and the coculture, but AG1024 inhibited the growth of DU-145 cells in the coculture with PrSC more strongly than that in the monoculture. These results indicate that IGF-I signaling is a valuable target for tumor-stromal interactions of prostate cancer.
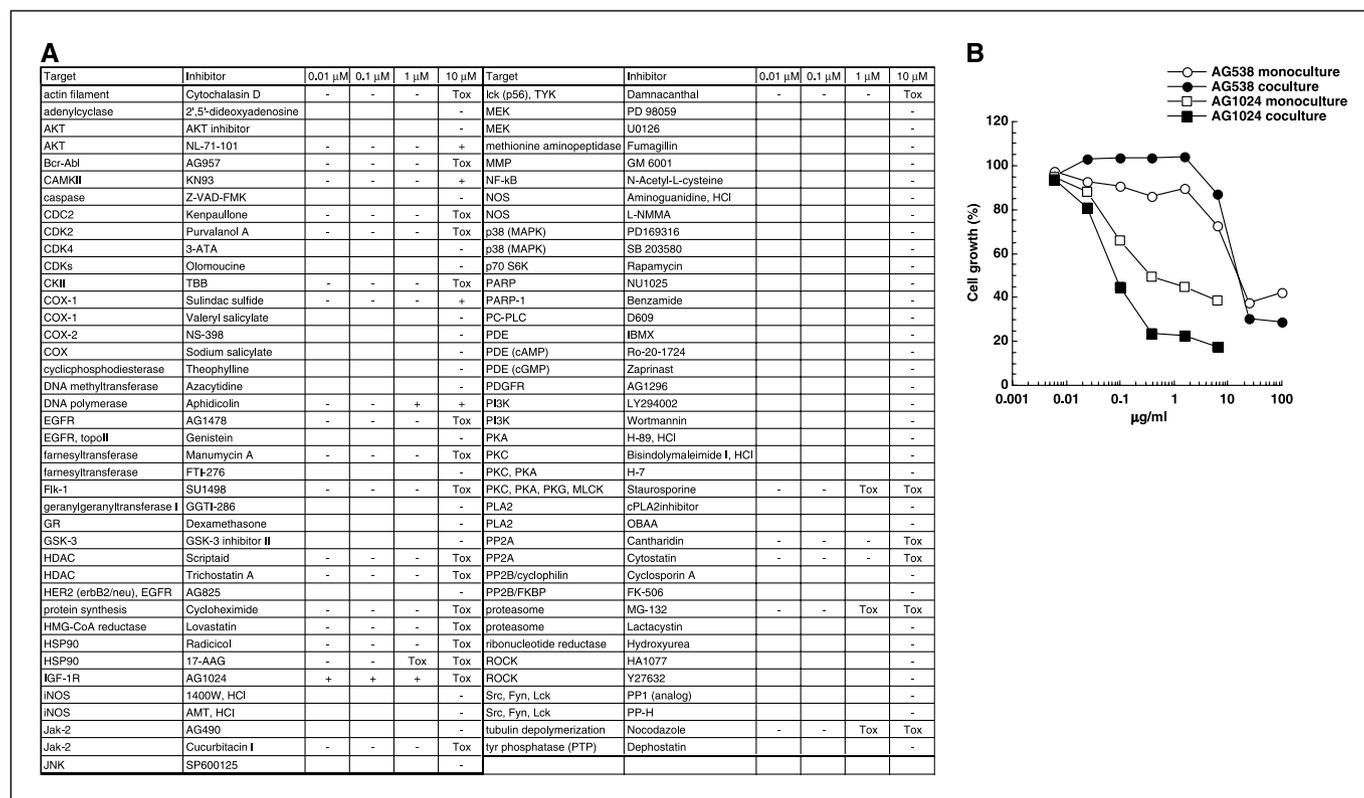
Discussion

We have previously established the *in vitro* coculture system of prostate cancer cells and PrSC and found that PrSC increased the growth of LNCaP and DU-145 cells (25). In this study, we first examined whether our previous *in vitro* finding reflects the *in vivo* nature. Actually, our present results showed that PrSC also increased the growth of LNCaP and DU-145 tumors *in vivo* (Fig. 1). Therefore, we used the *in vitro* system for the further studies. As we expected, the PrSC conditioned medium also increased the growth of LNCaP and DU-145 cells (Fig. 2), indicating that the secreted factors from PrSC modulated tumor growth. Our results revealed that one of the secreted factors is fibronectin (Fig. 2). Fibronectin is one of secreted extracellular matrix and increases the motility and growth of cancer cells (17, 34). Thus, it is considered that fibronectin partly increased the growth of LNCaP cells *in vivo* as well as *in vitro*.

In respect to IGFs actions, we found that IGF-I, but not IGF-II, transduced its signaling and increased the growth of LNCaP and DU-145 cells (Fig. 3). Our results were consistent with the others (5, 6). Although the growth stimulatory effect of IGF-I on LNCaP cells was

only seen in the presence of 5α-dihydrotestosterone, there are several reports describing the proliferative effect of IGF-I on LNCaP cells (6, 33). 5α-Dihydrotestosterone increases the expression of IGF-IR and enhances the sensitivity to the mitogenic effect of IGF-I on LNCaP cells indirectly (13). Therefore, the complex actions of fibronectin, IGF-I, and testosterone are considered to result in the significant increase of LNCaP tumors by PrSC in male SCID mice (Fig. 1). IGF-II is also reported to increase the growth of some prostate cancer cell lines (28, 35) but we did not obtain the same results (Fig. 3). It might be due to the affinity of IGF-II to IGF-IR being very low compared with IGF-I (2, 36). Although we did not detect IRS-I in LNCaP cells because of no expression of IRS-I (33), the IGF-I signaling is considered to be transduced by IRS-2 (9). Because the prostate cancer tissues show high expression of IGF-IR
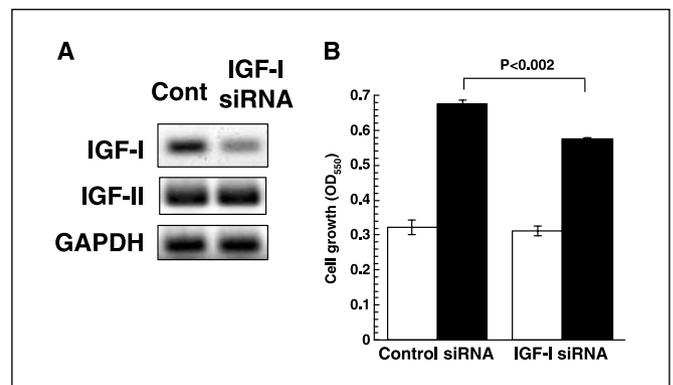
Figure 6. Effect of IGF-I siRNA on coculture. *A,* PrSC were treated with 100 nmol/L IGF-I siRNA or control siRNA for 2 days. Expressions of IGF-I and IGF-II were determined by RT-PCR. *B,* PrSC were treated with 100 nmol/L IGF-I siRNA or control siRNA for 2 days and then DU-145 cells were cocultured with the treated PrSC for 3 days (■). For monoculture of DU-145 cells (□), only medium without PrSC was treated with siRNAs as described above. The growth of DU-145 cells was determined using rhodanile blue dye. *Columns,* mean of triplicate determinations; *bars,* SD.

A

Target	Inhibitor	0.01 μM	0.1 μM	1 μM	10 μM	Target	Inhibitor	0.01 μM	0.1 μM	1 μM	10 μM
actin filament	Cytochalasin D	-	-	-	Tox	lck (p56), TYK	Damnacanthal	-	-	-	Tox
adenylcyclase	2',5'-dideoxyadenosine				-	MEK	PD 98059				-
AKT	AKT inhibitor				-	MEK	U0126				-
AKT	NL-71-101	-	-		+	methionine aminopeptidase	Fumagillin				-
Bcr-Abl	AG957	-	-	-	Tox	MMP	GM 6001				-
CAMKII	KN93	-	-	-	+	NF-kB	N-Acetyl-L-cysteine				-
caspase	Z-VAD-FMK				-	NOS	Aminoguanidine, HCl				-
CDC2	Kenpaullone	-	-	-	Tox	NOS	L-NMMA				-
CDK2	Purvalanol A	-	-	-	Tox	p38 (MAPK)	PD169316				-
CDK4	3-ATA				-	p38 (MAPK)	SB 203580				-
CDKs	Olomoucine				-	p70 S6K	Rapamycin				-
CKII	TBB	-	-	-	Tox	PARP	NU1025				-
COX-1	Sulindac sulfide	-	-	-	+	PARP-1	Benzamide				-
COX-1	Valeryl salicylate				-	PC-PLC	D609				-
COX-2	NS-398				-	PDE	IBMX				-
COX	Sodium salicylate				-	PDE (cAMP)	Ro-20-1724				-
cyclicphosphodiesterase	Theophylline				-	PDE (cGMP)	Zaprinast				-
DNA methyltransferase	Azacytidine				-	PDGFR	AG1296				-
DNA polymerase	Aphidicolin	-	-	+	+	PI3K	LY294002				-
EGFR	AG1478	-	-	-	Tox	PI3K	Wortmannin				-
EGFR, topoII	Genistein				-	PKA	H-89, HCl				-
farnesyltransferase	Manumycin A	-	-	-	Tox	PKC	Bisindolymaleimide I, HCl				-
farnesyltransferase	FTI-276				-	PKC, PKA	H-7				-
Flk-1	SU1498	-	-	-	Tox	PKC, PKA, PKG, MLCK	Staurosporine			Tox	Tox
geranylgeranyltransferase I	GGTI-286				-	PLA2	cPLA2inhibitor				-
GR	Dexamethasone				-	PLA2	OBAA				-
GSK-3	GSK-3 inhibitor II				-	PP2A	Cantharidin	-	-	-	Tox
HDAC	Scriptaid	-	-	-	Tox	PP2A	Cytostatin	-	-	-	Tox
HDAC	Trichostatin A	-	-	-	Tox	PP2B/cyclophilin	Cyclosporin A				-
HER2 (erbB2/neu), EGFR	AG825				-	PP2B/FKBP	FK-506				-
protein synthesis	Cycloheximide	-	-	-	Tox	proteasome	MG-132	-	-	Tox	Tox
HMG-CoA reductase	Lovastatin	-	-	-	Tox	proteasome	Lactacystin				-
HSP90	Radicicol	-	-	-	Tox	ribonucleotide reductase	Hydroxyurea				-
HSP90	17-AAG	-	-	Tox	Tox	ROCK	HA1077				-
IGF-1R	AG1024	+	+	+	Tox	ROCK	Y27632				-
iNOS	1400W, HCl				-	Src, Fyn, Lck	PP1 (analog)				-
iNOS	AMT, HCl				-	Src, Fyn, Lck	PP-H				-
Jak-2	AG490				-	tubulin depolymerization	Nocodazole	-	-	Tox	Tox
Jak-2	Cucurbitacin I	-	-	-	Tox	tyr phosphatase (PTP)	Dephostatin				-
JNK	SP600125				-						

B

Figure 7. Effects of various chemical inhibitors on coculture. *A,* DU-145 cells were cultured alone or cocultured with PrSC in the presence of the indicated concentrations of various inhibitors. +, the growth of DU-145 cells in the coculture with PrSC was inhibited strongly more than that in the monoculture; −, the growth of DU-145 cells was inhibited equally both in the coculture and the monoculture or not affected; blank, not tested. *B,* DU-145 cells were cultured alone (○, □) or cocultured with PrSC (●, ■) with the indicated concentrations of AG538 (○, ●) or AG1024 (□, ■) for 3 days. The growth of DU-145 was determined using rhodanile blue dye. *Points,* mean of duplicate determinations. Each SE is <10%.

(7, 8), they can respond to the action of IGF-I. Taken together, these results support the idea that the growth of prostate cancer cells that respond to IGF-I would be increased by the coculture with PrSC.

To ascertain the possibility that IGF-I secreted from PrSC modulates prostate cancer cell growth, we measured the IGF-I production in PrSC and the prostate cancer cell lines. As a result, PrSC were certainly found to express and secrete IGF-I (Fig. 4). Pietrzkowski et al. (37) reported that LNCaP and DU-145 cell lines produced substantial amounts of IGF-I, but others reported that the two cell lines did not secrete an immunoreactive level of IGF-I into their conditioned medium (6, 28). Our result showed that mRNA expression of IGF-I was detected in LNCaP cells, but the secreted levels of IGF-I from the two prostate cancer cell lines were very low compared with PrSC (Figs. 4 and 5). Therefore, it is considered that prostate cancer cells use IGF-I secreted by PrSC. In fact, it is shown that IGF-I mRNA and protein have been detected in the prostate stroma (7, 38). The level of IGF-I is increased dramatically in stroma surrounding the cancer cells, suggesting that the local stromal IGF-I may play an important role in cancer progression (39). Transforming growth factor β secreted from prostate cancer cells stimulates PrSC to differentiate to myofibroblasts and to express growth factors for cancer cell growth (40). We examined the possibility that prostate cancer cells modulate the secretion of IGF-I from PrSC. Whereas RT-PCR experiment showed a slight increase in mRNA expression of IGF-I in PrSC by the coculture with LNCaP and DU-145 cells, the secreted amounts of IGF-I were not increased by the coculture (Figs. 4 and 5). However, we cannot exclude the possibility that the secreted factors from

prostate cancer cells increase the secretion of IGF-I from PrSC, and we are investigating the possibility by the future study.

IGF-I has also been reported as a potent angiogenic factor and, very recently, as a lymphangiogenic factor (41). Whereas PrSC increased the growth of LNCaP and DU-145 tumors *in vivo* (Fig. 1), we did not detect apparent metastatic foci (data not shown). However, there is a possibility that PrSC increases angiogenesis and lymphangiogenesis in the tumors, in addition to the direct stimulation of tumor growth.

Between the two prostate cancer cell lines used in this study, DU-145 cells showed a better sensitivity to IGF-I. We used the DU-145 cell line to confirm that IGF-I is really involved in the tumor-stromal cell interactions of prostate cancer. Although the transfection efficiency of siRNA was only ∼ 10% in PrSC (data not shown), IGF-I siRNA significantly reduced IGF-I expression in PrSC and inhibited the growth of DU-145 cells in the coculture with the IGF-I siRNA–treated PrSC. Furthermore, the use of the SCADS inhibitor kit I consisting of 79 compounds with ∼ 60 different targets resulted in the finding that only IGF-IR tyrosine kinase inhibitor, AG1024, effectively inhibited the growth of DU-145 cells in the coculture with PrSC more than that in the monoculture (Fig. 7). These results clearly show that IGF-I secreted from PrSC increased the growth of prostate cancer cells in the coculture. Anti-IGF-I antibodies (42), anti-IGF-IR antibodies (43), antisense oligonucleotides to IGF-IR (12), and IGF-IR tyrosine kinase inhibitor (44) are reported to inhibit effectively the growth of some cancer cells *in vivo* including prostate cancer. These agents targeting the IGF-I signaling will be effective on tumor-stromal cell interactions mediated by IGF-I.

In conclusion, our present study has shown that IGF-I is really involved in the tumor-stromal interactions of prostate cancer and strongly suggested that the local secretion of IGF-I in prostate cancer tissue, in addition to the circulating IGF-I, is considered to play a critical role in the development of prostate cancer. Thus, this study greatly promotes the idea that the IGF-I signaling is a valuable target for the treatment of prostate cancer.

Acknowledgments

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Manabu Kawada, Hiroyuki Inoue, Tohru Masuda, et al.

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