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Short Communication

Proprotein convertases modulate budding and branching morphogenesis of rat ventral prostate

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ABSTRACT The onset of prostate morphogenesis is involved in the interaction between mesenchyme and epithelium. Proprotein convertases (PCs) activate a variety of growth and differentiation factors including mesenchymal and epithelial factors, such as insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β), which induce ductal budding and branching. In this study, we provide evidence that PCs play a critical role in prostatic budding from the urogenital sinus (UGS) and ductal branching morphogenesis of the neonatal rat ventral prostate. PCs were expressed only in the epithelial cells of neonatal rat prostate. PC activity in the ventral prostate was modulated by endogenous androgen. PC inhibition suppressed prostatic budding and branching. Taken together, our data indicates that androgen-induced PCs initiate the development of the prostate.

KEY WORDS: *proprotein convertase, prostate, development, furin, PC1*

Proprotein convertases (PCs) have been known as processing proteases that convert a wide variety of precursor proteins to their mature active form. To date, eight PCs have been identified including furin, PC1/3, PC2, PC4, PACE4, PC5/6 (and its isoform PC5B), PC7/8 and SKI-1. All PCs except for SKI-1 cleave precursor proteins at basic residues within the consensus motif (K/R)-$(X)_n$-(K/R)↓, when n=0, 2, 4 or 6 and X is usually not Cys. All PCs except for SKI-1 are responsible for processing of matrix metalloproteinases (MMPs), growth factors, membrane receptors and adhesion molecules (Khatib *et al.*, 2004). Therefore, these processing enzymes are suggested to have a fundamental role in embryogenesis, development and disease via the activation of their substrates (Thomas 2002).

The prostate is a male sex accessory gland and develops from the embryonic urogenital sinus (UGS). Prostatic buds emerge from urogenital sinus epithelium (UGE) and extend into urogenital sinus mesenchyme (UGM) where they undergo ductal branching morphogenesis under androgenic stimulation of interactions between mesenchymal and epithelial cells (Sugimura *et al.*, 1986). In the developing prostate, both growth and differentiation factors are derived from mesenchyme via androgenic stimulation which induce prostatic epithelial growth (Hayward *et al.*, 2000). As candidates of these factors, insulin-like growth factor (IGF-1), hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), bone morphogenetic protein-4 (BMP-4) and prostate differentiation factor (PDF) were reported previously (Hayward *et al.*, 2000, Lamm *et al.*, 2001, Paralkar *et al.*, 1998). Many growth and differentiation factors are first synthesized as larger biologically inactive precursors, which are proteolytically activated by PCs. Previously we reported that the inhibition of PC activity resulted in the loss of the luminal cell phenotype and induction of the basal cell phenotype in LNCaP (Uchida *et al.*, 2003). Our results suggest that PCs activity may be involved in the regulation of prostate epithelial cell differentiation. The role of PCs in the development of the prostate remains largely unknown. Therefore, we hypothesized that PCs regulate budding and branching morphogenesis of the prostate epithelium via activation of growth and differentiation factors.

In this study, we investigated the distribution of PCs and the effect of the PCs inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) on prostatic bud formation from the UGS and the development of rat ventral prostate.

Results and Discussion

PC expression in the UGS and neonatal rat prostate

All PCs except for PC4, which is confined to the testis and ovary, are expressed widely in a variety of tissues and cells (Hatsuzawa *et al.*, 1990). However, the expression, localization

Abbreviations used in this paper: CMK, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; IGF, insulin-like growth factor; PC, proprotein convertase; TGF, transforming growth factor; UGS urogenital sinus; VP, ventral prostate.

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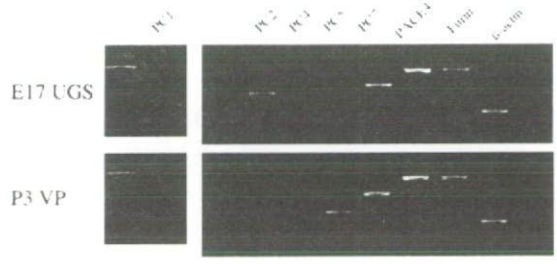


Fig. 1. Proprotein convertase (PC) mRNA expression in E17 urogenital sinus (UGS) and P3 ventral prostate. *Amplification products for PC1 and other PCs were separated by 2 % and 4 % agarose gel electrophoresis, respectively. PC2 mRNA was detected in the E17 UGS, not in the P3 ventral prostate*

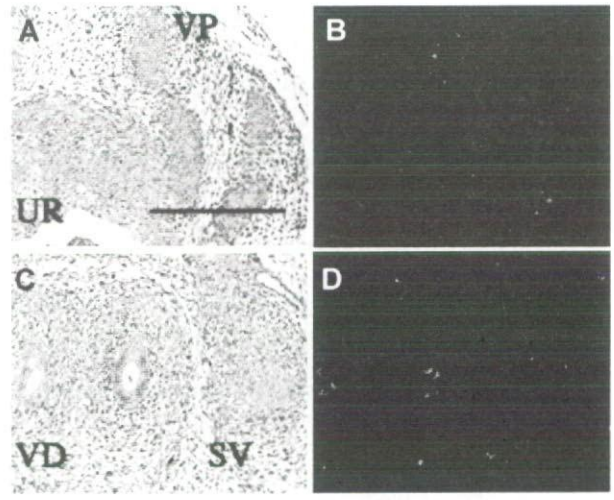


Fig. 2. PC1 distribution in the developing prostate. *Light micrographs of HE staining of P3 rat prostate* **(A,C)** *and immunostaining for PC1* **(B,D)**. *PC1-positive cells were detected in the epithelial cells of the VP (B), SV and VD (D). PC1 staining was not observed in the epithelial cells of the urethra and mesenchymal cells of the prostate. Abbreviations: SV, seminal vesicle; UR urethra; VD, vas deferens; VP, ventral prostate. Bar, 200 μm.*

and function of PCs in the prostate are still unknown. We first examined the gene expression and protein distribution of PCs in the developing prostate. Gene expressions of all PCs except for PC4 were detected in E17 UGS by RT-PCR. In contrast, PC2 and PC4 gene expression was not detected in P3 VP, whereas mRNAs of PC1, PC5, PC7, furin and PACE4 were observed (Fig. 1). Next we confirmed PCs expression and distribution by immunofluorescence staining. We examined the distribution of furin and PC1 in the developing prostate by immunofluorescence staining. At P3, cytoplasmic staining of PC1 was observed in the epithelial cells of the prostate, seminal vesicle and vas deferens. PC1 immunoreactivity was not observed in the mesenchymal cells of the prostate (Fig. 2). PC1 was also detected in endothelial cells and red blood cells. The distribution of furin-positive cells was similar to that of PC1-positive cells (data not shown). Furin and PC1 expressions were not observed in urethral mesenchyme and epithelium. At E17, furin and PC1 were detected in the epithelial cells of the vas deferens, but not epithelial cells of the UGS (data not shown). The discrepancy in PCs expression at UGS between RT-PCR and immunofluorescence staining could be due to the lower expression of PCs or the contamination of the vas deferens.

The modulation of PC activity in the neonatal ventral prostate

Previous studies demonstrated that androgenic effects on epithelial growth, budding and ductal branching morphogenesis of the prostate were mediated by mesenchymal androgen receptors (Cunha *et al.*, 1980). Therefore, we hypothesized that the activity of epithelial PCs could be regulated by testosterone via epithelial-mesenchymal interaction. To investigate regulation of PCs activity by testosterone, E17 UGS and P1 VPs were cultured with or without testosterone. Comparable cultures were treated with CMK, the PC inhibitor. Results demonstrated that PCs activity was significantly increased by the addition of testosterone and PCs activity induced by testosterone was significantly inhibited by CMK at 50 μM in P1 VP (Fig. 3B), but not in E17 UGS (Fig. 3A).

Our results suggested that PC were expressed in the epithelium of the prostate during the period of budding and branching morphogenesis and that the activity of epithelial PCs was regulated by testosterone via epithelial-mesenchymal interaction. Our results also showed that testosterone treatment had minimal effect on P1 VP and did not affect PCs activity in UGS. These could be due to the lower activity of specific PC modulated by

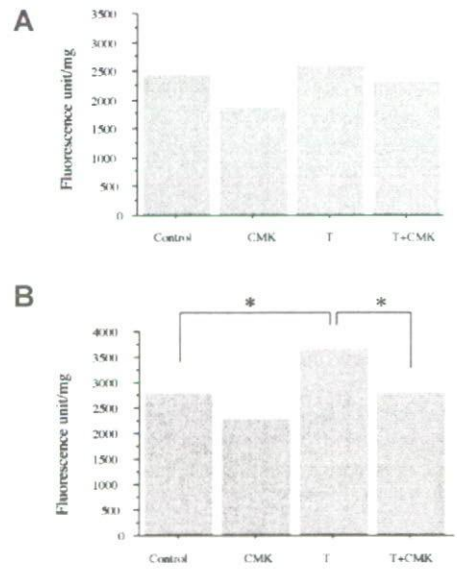
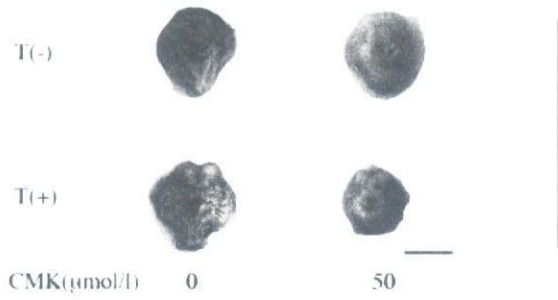


Fig. 3. Proprotein convertase (PC) activity as measured by boc-RVRR-AMC processing. **(A)** *The effect of 10 nM testosterone and 50 μM CMK on PC activity in the urogenital sinus. No significant differences were observed.* **(B)** *The effect of 10 nM testosterone and 50 μM CMK on PC activity of the rat P1 ventral prostate. PC activity was significantly increased by the addition of 10 nM testosterone. Testosterone-induced PC activity was significantly decreased by 50 μM CMK. *p < 0.05*



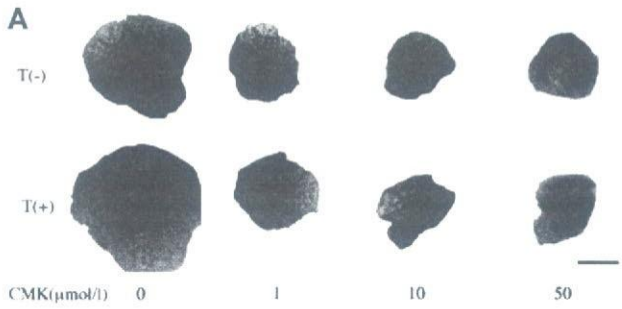


Fig. 4. Effect of the proprotein convertase inhibitor, CMK, on prostatic bud formation. *Representative images of rat urogenital sinus (UGS) cultured for 4 days in serum free medium without testosterone and CMK, with 10 nM testosterone alone, with both 50 μM CMK +10 nM testosterone and 50 μM CMK alone. Prostatic budding was observed by androgen stimulation. Androgen-stimulated budding was inhibited by treatment with 50 μM CMK. Bar, 1 mm.*

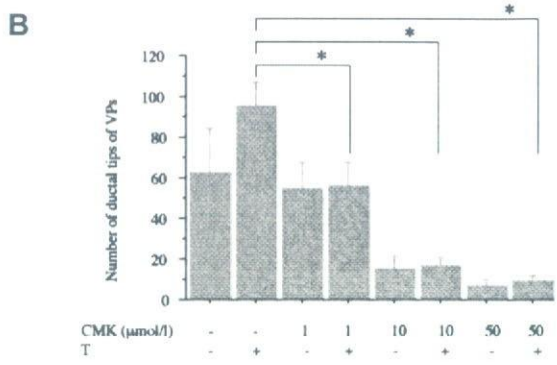


Fig. 5. Effect of the proprotein convertase inhibitor, CMK, on branching morphogenesis. (A) *Representative images of rat P1 ventral prostates cultured for 6 days in serum free medium with/without 10 nM testosterone, with various concentrations of CMK. Prostatic ductal growth and branching were observed by androgen stimulation. Androgen-stimulated growth was inhibited by treatment with CMK in a dose-dependent manner. Bar, 2 mm.* **(B)** *Quantification of branching with CMK treatment. Branching was significantly increased with testosterone and significantly inhibited by the addition of CMK in a dose-dependent manner. *p < 0.05*

testosterone or the selectivity of boc-RVRR-AMC for specific PC, because wide variety of PCs was expressed in VP and UGS.

Effect of the proprotein convertase inhibitor, CMK, on prostatic bud formation and branching morphogenesis

We next focused the effect of PCs on the prostatic budding and branching morphogenesis using *in vitro* organ culture system. Rat E17 UGS were cultured for 4 days and P1 VPs for 6 days. In the presence of testosterone at 10 nM, prostatic buds emerged from the UGE and elongated into the UGM. Testosterone-induced prostatic budding was inhibited by the addition of 50 μM CMK (Fig. 4). Neonatal VPs grew and underwent ductal brunching morphogenesis extensively with 10 nM testosterone. The addition of CMK inhibited the growth of neonatal prostate and significantly induced the reduction of the number of ductal tips with or without 10 nM testosterone in a dose- and a time-dependent manner (Fig 5A, B).

Histological and immunohistochemical analysis of cultured VPs was examined. Microscopically, the higher frequency of canalization was observed in the prostate treated with testosterone alone, not in the prostate with CMK (Fig. 6A). The diameter of ducts was significantly increased by the addition of CMK (Fig. 6B). In the prostate treated with testosterone alone, nuclear staining of PCNA was more frequently observed in the ductal tip than in the base of the prostate by immunohistochemical staining. In contrast, PCNA expression was frequently observed in the tip and base of the prostate treated with testosterone + CMK (Fig. 6C).

TABLE 1

PRIMERS USED FOR RT-PCR AMPLIFICATION

Gene	Primers	fragment size (bp)	Reference
PC1/3	S* 5'-GCTACTAATCTCACTCAAAGC-3'	143	Lee 1999
	AS 5'-CCTTCTCTTAATATGCCAAC-3'		
PC2	S 5'-GCTAGACTTGAATGTGGC-3'	525	Rovere 1996
	AS 5'-GTTGCAGTCATCGTAGCT-3'		
PC4	S 5'-AGCCGCAACACATACACATC-3'	680	
	AS 5'-GCCATCGCAGCATACAGTCA-3'		
PC5	S 5'-CTGCTGGTTTAAAGGTGAGCCA-3'	403	Akamatsu 2000
	AS 5'-TCACCAGCAGCTCTTTCTCC		
PC7	S 5'-GCCCAGGAAGAGACATCAAT-3'	640	
	AS 5'-CATCTTGTCTCCTCCACTGA-3'		
PACE4	S 5'-CCCTCTGGAACCAAGTCTCAACTT-3'	1041	Akamatsu 2000
	AS 5'-TGAAGCCAGCTTTACATCTGCTGC-3'		
Furin	S 5'-AATGGTGTCTGTGGTGTAGG-3'	1050	
	AS 5'-GTCAGCGTCCCATAGTTGTT-3'		

*S, sense; AS, antisense

Prostatic development is characterized by the bud formation, canalization and branching mediated via the epithelial-mesenchymal interaction under the balance of stimulatory and inhibitory factors. Among PCs substrates, IGF-1 and HGF are considered to be responsible for prostatic development as the stimulatory factors and BMP-4 is known as the inhibitory factor. TGF-β has the stimulatory effects on the periphery and inhibitory effects on the center of the prostate (Tomlinson *et al.*, 2004). In addition, previous study demonstrated that MMPs and integrins, which were considered as PCs substrates, also had the stimulatory effect on the branching of mammary gland and ureteric bud (Simian *et al.*, 2001, Pohl *et al.*, 2000, Zent *et al.*, 2001). PCs inhibition might cause an imbalance in the activation of stimulatory and inhibitory substrates. In fact, our macroscopic observa-

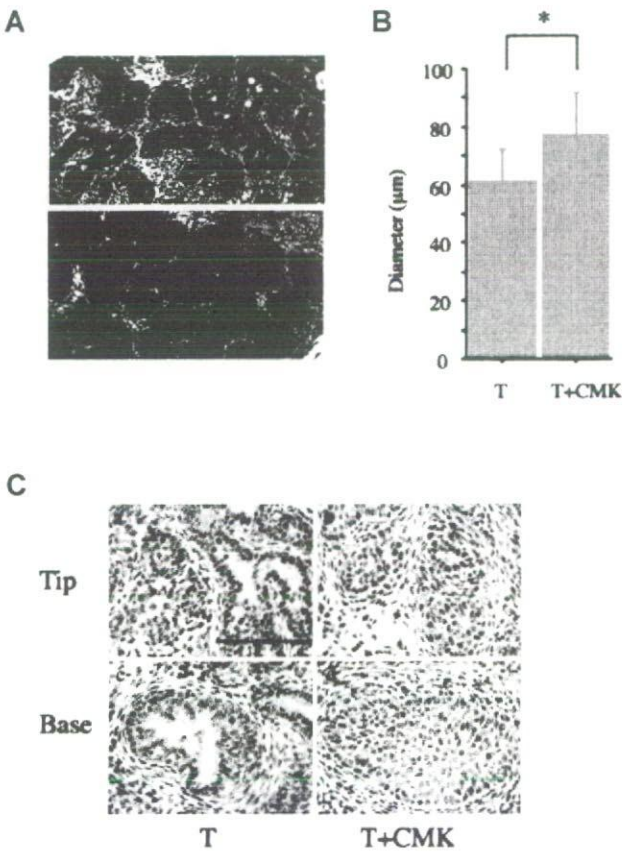


Fig. 6. Effect of the proprotein convertase inhibitor, CMK, on branching morphogenesis – microscopic and immunohistochemical analysis. (A) *Representative microscopic images (HE staining) of rat P1 ventral prostates cultured for 6 days in serum free medium with 10 nM testosterone, with/without 10 μM CMK. The higher frequency of canalization, the lower cell density of epithelium was observed in the prostate treated with testosterone without CMK (a), not in the prostate with CMK (b). Bar, 200 μm.* **(B)** *The diameter of ducts with testosterone, with/without CMK. The diameter was significantly increased by the addition of CMK. *p < 0.05.* **(C)** *Immunostaining for PCNA of the prostate treated with testosterone alone (a, c) and with testosterone and CMK (b, d). PCNA expression was observed in the ductal tip (a, b) and base (c, d). Bar, 200 μm.*

tion showed that PCs inhibitor reduced prostatic budding and branching, suggesting that BMP-4 activation might be accelerated by the inhibition of PCs. On the other hand, microscopic observation showed that PCs inhibitor induced the enlargement of the prostatic epithelial cord and the reduction of canalization. Thease results could be associated with the inactivation of morphogens, such as TGF-β and PDF, by the inhibition of PCs. In addition, PCNA expression indicated that PCs inhibitor initiated the epithelial proliferation in the base, which might represent the imbalance of the prostatic growth and be resulted in the enlargement of the prostatic epithelial cord. Our results indicate PCs are involved in the usual process of the prostatic development, including the budding, cord formation, canalization and branching. Therefore, the difference of the phenotypic expression in the prostate treated with or without PCs inhibitor would be important. The expression and localization of each PC, by which substrates are predominantly activated and responsible for the each steps of prostatic development, should be studied further.

In conclusion, our results suggest an important role of PCs in the prostatic morphogenesis. PCs modulated by androgen are expressed in neonatal VP and may be responsible for the processing of mesenchymal and epithelial factors, which initiate budding and branching of the prostate.

Materials and Methods

Materials

The animal use procedures were in accordance with the guide for the care and use of laboratory animals of Mie University Graduate School of Medicine. Sprague-Dawley (SD) rats were obtained from CLEA (Tokyo, Japan). Testosterone, insulin and transferrin were obtained from Sigma at MO. DMEM-Ham's F12 was purchased from Invitrogen (Carlsbad, CA), gentamycin from Wako (Osaka, Japan). N-t-butoxycarbonyl-Arg-Val-Arg-Arg-7-amino-4-methylcoumarine (Boc-RVRR-AMC) was obtained from Bachem (King of Prussia, PA) and decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) from Sigma (St. Louis, MO). Anti-furin and anti-PCNA antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-PC1 antiserum was kindly gifted by Dr. Tanaka at Shizuoka University in Japan. FITC-labeled secondary antibody was purchased from MBL (Nagoya, Japan).

Tissue collection

Rat UGS and VPs were obtained from SD rats. The day of detection of a vaginal plaque and the day of birth was designed E0 and P0, respectively. UGS from E17 rats and VPs from P0 and P3 neonatal rats were dissected and used for immunofluorescence staining, PC activity assay and *in vitro* organ culture.

RT-PCR Analysis

Total RNA was prepared from E17 UGS and P3 VP using RNeasy (Qiagen, CA). cDNA was prepared using Superscript (Invitrogen, CA). The efficiency of each cDNA reaction was assayed by amplification of β-actin transcripts with primers for β-actin (R & D systems, MN). Primers used to amplify PCs gene were shown in Table1. The viability of the primer for PC4 was conformed using neonatal rat testis. The first strand cDNA was amplified with amplitaq gold (Applied biosystems, NJ). The PCR condition was as follows: 1 cycle at 95°C at for 5 min; 35 cycles at 95°C for 15 s, 53-57°C for 30 s, 72°C for 1 min 30 s. Amplification products (5 μl) were separated by 2-4% agarose gel electrophoresis.

Immunofluorescence staining

Dissected E17 UGS and P3 prostate were fixed with 10% formaldehyde for 3 h on ice, processed into paraffin and then sectioned at 3 μm. Sections were deparaffinized with Histoclear. To enhance immunoreactivity for PCs, sections were oxidized before immunostaining with Gomori's oxidation mixture, as described previously (Kurabuchi and Tanaka, 1997). Rat pancreas was used as the positive control. Nonspecific binding was blocked with blocking buffer (DAKO, CA) for 1 hour at room temperature. Sections were sequentially incubated overnight at 4°C with following reagents: anti-furin antibody (1:50), anti-PC1 antiserum (1:500). The next day, sections were rinsed and incubated in a FITC-labeled secondary antibody and processed according to the manufacture's instructions.

Proprotein convertase activity assay

E17 UGS and P1 VP were cultured with and without 10 nM testosterone and 50 μM CMK. After 24 h incubation, UGS and VP were washed with PBS and incubated with assay medium which consisted of the growth medium containing 0.25 % Triton X-100 to permeabilize cells and boc-RVRR-AMC (100 μM) as a fluorogenic substrate. Fluorescence was

measured at 360 nm excitation and 460 nm emission wavelengths after 4 h of substrate addition. Tissue weights of UGS and VPs were measured for normalization before the addition of assay medium. The data were normalized to tissue weights

In vitro *organ culture*

UGS from E17 rats and VPs from P1 rats were grown in an organ culture system as previously described with modifications (Sugimura *et al.*, 1996). Briefly, dissected tissues were placed on Millicell membranes (Millipore, Bedford, MA) in 6-well plates (BD bioscience, Franklin Lakes, NJ) and cultured in DMEM-Ham's F12 without phenol red supplemented with insulin (10 μg/ml), transferrin (10 μg/ml) and gentamycin (50 μg/ml) at 5% CO_2. Testosterone (10 nM) and various concentrations of CMK were added and the medium was changed every day. Individual ductal tips were counted manually. Four to five VPs or UGSs were used for each group in one experiment. The experiment was repeated at least three times.

Histological and immunohistochemical analysis of cultured VPs

The prostates cultured with 10 nM testosterone with/without 10 μM CMK, were processed into paraffin and then sectioned at 3 μm. Sections were deparaffinized with Histoclear, used for Hematoxylin-Eosin (HE) staining and immunohistochemical staining. The diameter of the prostattic duct was measured under light microscope. Immunohistochemical staining was performed with Vectastain ABC kit (Vectastain Laboratories, CA). Briefly, sections were incubated overnight at 4°C with following reagents: anti-PCNA antibody (1:5000), The next day, sections were rinsed and incubated in a secondary antibody and processed.

Statistical analysis

Data are presented as the mean SD of four to five samples in each of two to six independent trials. Statistical analysis was performed using the Student *t* test. P value < 0.05 was considered significant.

Acknowledgements

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Naftopidil, a selective α-1 adrenoceptor antagonist, inhibits growth of human prostate cancer cells by G1 cell cycle arrest

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α-1 adrenoceptor antagonists are generally prescribed for benign prostate hyperplasia with lower urinary tract symptoms. Naftopidil, a selective α-1 adrenoceptor antagonist, is frequently used in Japan because it has fewer side effects. Here we demonstrate for the first time that naftopidil has growth inhibitory effect in androgen-sensitive and -insensitive human prostate cancer cell lines. The concentrations causing 50% inhibition (IC50) of cancer cell growth were 22.2 ± 4.0 μM in androgen-sensitive LNCaP cells and 33.2 ± 1.1 μM in androgen-insensitive PC-3 cells. FACS analysis revealed that cell growth inhibition by naftopidil was due to the arrest of the G1 cell cycle. Expressions of $p27^{kip1}$ and $p21^{cip1}$ were significantly increased in LNCaP cells treated with naftopidil. In PC-3 cells, naftopidil induced $p21^{cip1}$ but not $p27^{kip1}$. *In vivo*, oral administration of naftopidil to nude mice inhibited the growth of PC-3 tumors as compared to vehicle-treated controls. These results suggest that naftopidil may be useful in the chemoprevention of prostate cancer and the intervention of hormone refractory prostate cancer.

Key words: prostate cancer; naftopidil; cell cycle

Prostate cancer is the third most common cancer in the world and sixth leading cause of cancer death in males.[1] The incidence and mortality of prostate cancer are still lower in Japan than in western countries, but both rates are rapidly increasing recently. Age-adjusted incidence and mortality of prostate cancer have risen from 7.1% in 1975 to 19.9% in 1998, and from 0.5% in 1950 to 8.6% in 2000.[2] In regard to treatment, organ-confined prostate cancer is curable with surgery or radiotherapy, but management of non-organ-confined disease fails in many cases.[3] For non-organ-confined disease, especially metastatic prostate cancer, androgen ablation therapy has been the standard therapy.[4] Generally, activation of the apoptotic pathway causes tumor shrinkage in androgen-sensitive prostate cancer cells; however, in androgen-insensitive prostate cancer cells, the apoptotic pathway is not activated by androgen ablation therapy.[5] When prostate cancer is clinically diagnosed, both androgen-sensitive and androgen-insensitive prostate cancer cells are present.[6,7] Proliferation of androgen-insensitive prostate cancer cells causes prostate cancer death[5,6] and conventional therapies for hormone-refractory prostate cancer (HRPC) have done little to improve the treatment outcomes or survival rates.[8] Therefore, early detection and elimination of both types of prostate cancer cells are important for decreasing prostate cancer-related death.

Another common prostatic disease in aging males is benign prostate hyperplasia (BPH). The incidence is very high: 40% among men in their 50s and 70% among men in their 60s.[9] In addition, about 50% of men over 50 years of age have BPH-related lower urinary tract symptoms (LUTS) requiring treatment.[10] Because of the aging populations in western countries and the Japanese population in general, and the 'baby boomers' in particular, management and treatment of BPH are becoming more important. α-1 adrenoceptor antagonists, which reduce prostatic smooth muscle tone and exert immediate effect on urinary flow, are the most frequently prescribed therapeutic agents for BPH patients with LUTS.[11,12] α-1 adrenoceptors are divided into α-1A, -1B and -1D subtypes.[13] In the prostate gland, α-1D adrenoceptor is the second most abundantly expressed subtype after α-1A.[14] Naftopidil is an α-1 adrenoceptor antagonist with higher selectivity for the α-1A and -1D subtypes, and especially high affinity to the latter.[15] In Japan, like tamsulosin, naftopidil is prescribed widely for patients with LUTS associated with BPH.[16] Several reports demonstrated that naftopidil improved bladder capacity and relaxed voiding *via* inhibition of afferent nerve activity,[17,18] and clinically improved not only obstructive voiding symptoms but also storage symptoms associated with BPH.[19,20]

Kyprianou and Benning demonstrated that doxazosin and terazosin (quinazoline-derived α-1 adrenoceptor antagonists) had growth-inhibitory effects in human prostate cancer cell lines.[21] Doxazosin induced apoptosis in prostate cancer cells, although that action was independent of the capacity to antagonize α-1 adrenoceptors. Because α-1 adrenoceptor antagonists are prescribed for aging male patients with BPH, these might also be ideal chemopreventive agents. Recently, Harris *et al.* showed that treatment with quinazoline-based α-1 adrenoceptor antagonists decreased the relative risk of prostate cancer development.[22] However, the non-selective α-1 adrenoceptor antagonists doxazosin and terazosin have more cardiovascular adverse effects than selective agents,[23] and these adverse effects most frequently lead to treatment discontinuation.[24] High tolerability is important for continued administration of drugs, and uro-selective α-1 adrenoceptor antagonists may have advantage in this regard. In contrast, several evidences demonstrate that tamsulosin, an uro-selective agent, has no effect on prostate cancer cell proliferation.[21,25]

The principle aim of our study was to determine whether naftopidil has growth inhibitory effect on prostate cancer. To conduct our study, we used androgen-sensitive and -insensitive prostate cancer cell lines, LNCaP and PC-3, respectively. The molecular mechanisms involved in the anti-proliferative effect of naftopidil were studied in both cell lines. Moreover, *in vivo* anti-tumor activity was investigated in a PC-3 xenograft model.

Material and methods

Materials

Naftopidil was kindly provided by Nippon Organon K.K. (Osaka, Japan). Antibodies against CDK2 (M2), CDK4 (c-22), cyclin D1 (R-124), cyclin E (E-5) and Ki-67 (Ki-67) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); against p16INK4a (G175-405), p27kip1 (57), p21cip1 (6B6), p53 (DO-7) and CD31/PECAM-1 (MEC13.3) from BD Pharmingen (San Diego, CA); and against Akt and phospho-Akt from Cell Signaling Technology (Beverly, MA). Human prostate total RNA (No. 7,988) was purchased from Ambion (Austin, TX).

Cell culture

Human prostate cancer cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Rockville, MD). These cancer cell lines were maintained in RPMI-1640

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medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Tokyo, Japan) and 100 U/ml penicillin and 100 μg/ml streptomycin in a water-jacketed incubator with a humidified atmosphere (5% CO_2, 95% air) at 37°C.

Cell viability assay

All cell lines were plated in 12-well multi-dishes (2×10^4 cells/well). One day (PC-3) or 2 days (LNCaP) later, the cells were treated with increasing concentration of naftopidil or vehicle (0.1% DMSO) for 3 days (PC-3) or 4 days (LNCaP). Then, the cells were detached by trypsinization and counted using a hemocytometer. Cell viability was assessed by trypan blue exclusion assay and expressed as percentage over the control.

Cell cycle analysis

Cell cycle analysis was performed by flow cytometry. Cells were treated with either 20 μM naftopidil (LNCaP), 40 μM naftopidil (PC-3) or vehicle (0.1% DMSO) for 24 hr, then trypsinized and washed once with phosphate-buffer saline (PBS), fixed in 70% ethanol and stored at 4°C for subsequent cell cycle analysis. Fixed cells were washed with PBS and incubated with PBS containing 20 μg/ml RNaseA and 0.3% NP-40 for 30 min at 37°C, then stained with 50 μg/ml propidium iodide (PI) for 30 min at 4°C in the dark. The DNA content of $\sim 1 \times 10^6$ stained cells was analyzed on a Becton Dickinson FACS Calibur (Mansfield, MA) flow cytometer. The fractions of cells in the G0/G1, S and G2/M phases were calculated using Cell Quest software (Becton Dickinson, Mansfield, MA).

Western blot analysis

The cells were rinsed with ice-cold PBS and homogenized in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% NP-40, 1% Protease inhibitor [Nakarai, Tokyo, Japan]) for 30 min at 4°C. After centrifugation at 10,000g for 10 min at 4°C, the supernatant (total cell lysate) was collected and frozen at −20°C until analysis. The protein concentrations were measured using Bio-Rad protein assay reagent (Hercules, CA). For western blot analysis, protein samples were boiled for 5 min in Laemmli buffer (62.5 mM Tris [pH 6.8], 2% SDS, 20% glycerol, 0.01% bromophenol blue, 100 mM DTT) and separated on 7.5% or 12.5% polyacrylamide gel electrophoresis (ATTO, Tokyo, Japan). The gels were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) according to a semidry transfer method. The membranes were blocked with Detector Block Solution (KPL, Gaithersburg, MD) for 1 hr at room temperature and incubated with primary antibody overnight at 4°C. After washing 3 times in Tris-buffered saline/Tween 20 (TBS-T), the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hr at room temperature. After washing 3 times in TBS-T, antibody positive bands were visualized using chemilumiescence (ECL) western blot detection reagents (Pierce Biotechnology Rockford, IL).

Isolation of total RNA and RT-PCR amplification

Total RNA was extracted from each cell line using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from 3 μg of total RNA by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a total volume of 20 μl. RT-PCR was performed using 2 μl of RT products with Takara Ex Taq (Takara, Tokyo, Japan) in accordance with the manufacturer's protocol. The PCR primers for amplification of α-1 adrenoceptor mRNA subtypes were as follows: 5′-ATATACCC ATGCTCCAGC-3′ and 3′-GCTTTTACTTCTCACCCG-5′ (52°C) for α-1A; 5′-GCTCCTTCTACATCCCTCTGG-3′ and 3′-AGGGT AGCCAGCACAAGATGA-5′ (60°C) for α-1B; 5′-CTACG AATTGGCCGACT-3′ and 3′-GGATGGGGCAGTGTTTC-5′ (54°C) for α-1D. For β-actin mRNA analysis, primers were 5′-GAGAAGATGACCCAGATCATGT-3′ and 3′-TCGTCATACTC CTGCTTGCTG-5′ (60°C). PCR reactions were denatured at 94°C for 1 min, in 30 cycles (β-actin) and 35 cycles (α-1 adrenoreceptors) at 95°C for 30 sec; each annealing temperature was initially held for 30 sec and then 72°C was held for 30 sec, followed by a final extension at 72°C for 4 min. Amplified products were separated on 1.5% ethidium bromide-stained agarose gel and photographed under UV light.

ELISA assay for prostate-specific antigen

LNCaP cells were treated with various concentrations of naftopidil for 4 days as described earlier. The conditioned media from these LNCaP cells were assayed for prostate-specific antigen (PSA) secretion by use of a PSA Enzyme Immunoassay test kit (Hope Laboratories, Belmont, CA).

In vivo *tumor growth*

The anti-tumor activity of naftopidil was determined in athymic nude mice bearing PC-3 cells. PC-3 cells (2×10^6) mixed with Matrigel (Becton Dickinson, Mansfield, MA) were injected into the flank region of male athymic nude mice (4–6 weeks old), and the mice were maintained in a pathogen-free environment. Treatments were started 7 days after injection of PC-3 cells: by that time, all mice had developed palpable tumors. Mice were divided into 3 groups ($n = 10$ each): Group 1, vehicle; group 2, treatment with 10 mg/kg/day naftopidil; Group 3, treatment with 100 mg/kg/day naftopidil. Naftopidil was administered orally (10 ml/kg/day) as a suspension in 0.5% carboxymethylcellulose through a 22-gauge, 1.5-inch gavage needle for 28 days. Tumors were measured with a caliper every 7 days and tumor volumes were calculated by the formula: $(\text{width})^2 \times \text{length} \times 0.5$.

Immunohistochemistry

Tissue samples from PC-3 tumors were analyzed for altered patterns of Ki67, $p21^{Cip1}$, microvessel density (MVD) and apoptosis between control and naftopidil-treated animals. Tissues fixed in formalin or zinc (for MVD) were dehydrated and embedded in paraffin. Four micrometer sections were cut using a Leica RM2125 rotary microtome (Leica Microsystem, Wetzelar, Germany). After deparaffinization, sections were hydrated and incubated for 20 min in 0.3% hydrogen peroxide in methanol. For antigen retrieval, sections were heated in a microwave oven in 0.01 M citrate buffer, pH 6.0, to 100°C for 10 min. After a period of cooling, nonspecific binding was blocked using blocking serum (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Primary antibodies were incubated overnight at 4°C, and then incubated with diluted biotynylated secondary antibody solution at room temperature for 30 min. After washing, sections were incubated with Vectastain (Vector Laboratories, Burlingame, CA) at room temperature for 60 min. Visualization of immunostaining was achieved using 3,3′-diaminobenzidine (DAB). After a rinse in running water, sections were stained with hematoxylin. Anti-Ki67, $p21^{Cip1}$ and CD31 were used at dilutions of 1:20, 1:100 and 1:100, respectively. Apoptotic cells were labeled by the TUNEL method using TACS 2 TdT-DAB *In situ* Apoptosis Detection Kit (Travigen, Gaithersburg, MD) following the manufacture's instruction. The expression levels of Ki67, $p21^{Cip1}$ and apoptosis were determined by the percentage of positive nuclear cells of 10 separate microscopic fields (400×) from each tissue specimens. MVD were assessed by counting CD31 positive microvessels on a 200× field (0.74 mm^2/field) of 10 separate areas. The results were independently reviewed by 2 blinded investigators.

Statistical analysis

The results were expressed as the means ± SD. Data were analyzed using Student's *t*-test and Fisher's PLSD was used for between groups comparison. $p < 0.05$ was considered statistically significant.

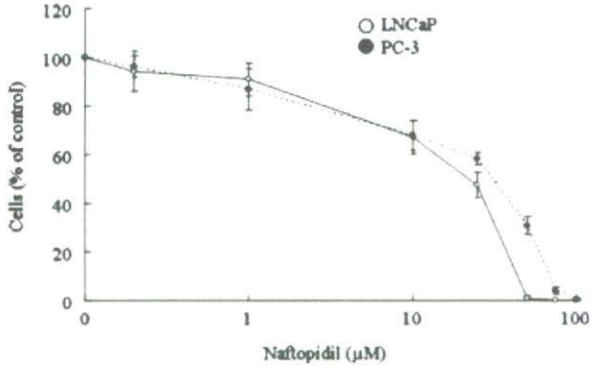

FIGURE 1 – Dose-dependent inhibitory effect of naftopidil on human prostate cancer cell proliferation. The cells were exposed to various concentration of naftopidil (0, 0.5, 1, 10, 25, 50, 75 and 100 μM) for 3 days (PC-3) or 4 days (LNCaP). Values represent the mean ± SD percentage of viable cells from 3 separate experiments (in duplicate). LNCaP: open circles and PC-3: closed circles.

Results

Antiproliferative effect of naftopidil on prostate cancer cells

The human prostate cancer cell lines LNCaP and PC-3 were treated with various concentrations of naftopidil in medium containing 10% FBS, and the effect of naftopidil on cell proliferation was determined after 3 days (PC-3) or 4 days (LNCaP) of incubation. As shown in Figure 1, naftopidil inhibited the proliferation of both LNCaP and PC-3 cells in a dose-dependent manner. The concentrations causing 50% inhibition (IC50) in LNCaP and PC-3 were 22.2 ± 4.0 μM and 33.2 ± 1.1 μM, respectively.

Induction of cell cycle arrest at G1 phase by naftopidil in prostate cancer cells

The proliferation-inhibitory mechanism by naftopidil was investigated flow- cytometrically in PI-stained cells. In comparing with vehicle, naftopidil increased the population of cells in the G1 phase, and decreased populations of cells in phases S and G2 (Fig. 2).

Effects of naftopidil on the expression of positive and negative cell cycle regulators

To determine which key cell-cycle regulators are involved in naftopidil-induced G1 arrest, we investigated the expressions of

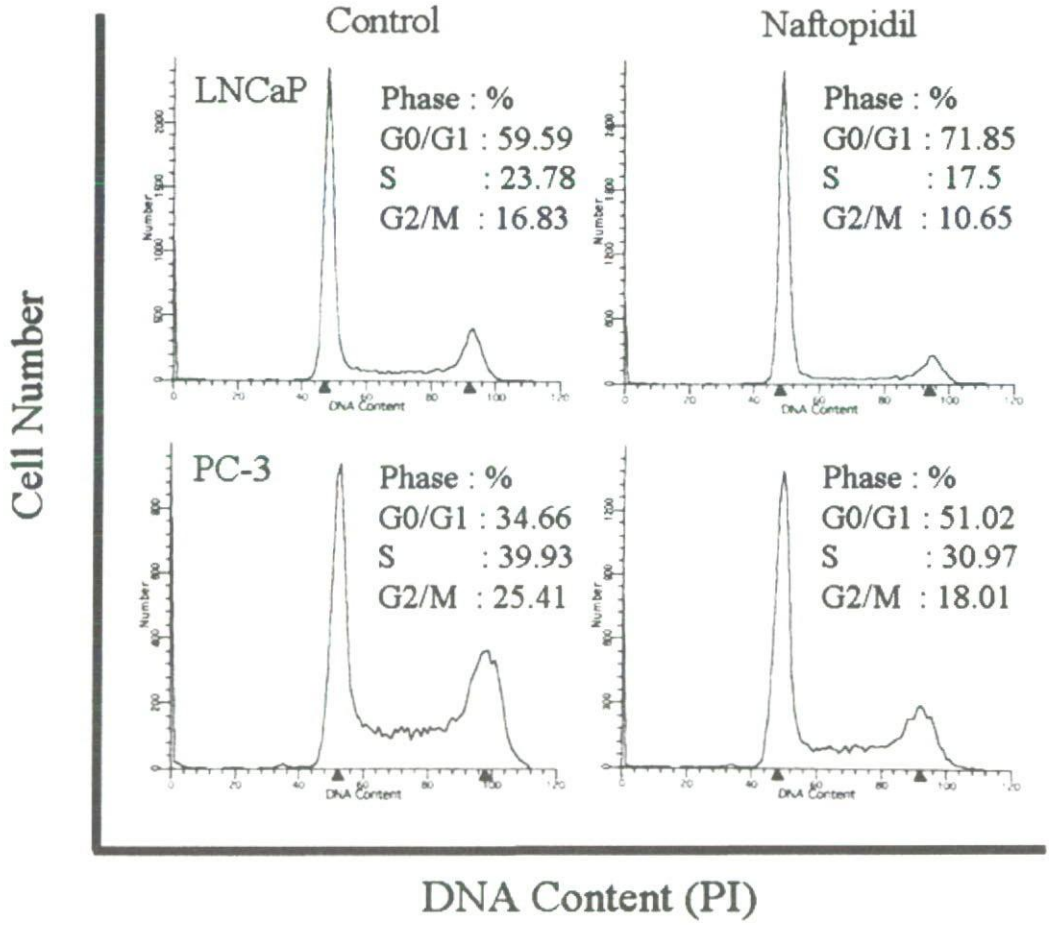

FIGURE 2 – Cell cycle analysis by flow cytometry of prostate cancer cells treated with naftopidil. The cell cycle was determined by propidium iodide (PI) staining as detailed in 'Material and methods'. The proportions of cells in G0/G1, S and G2/M phase are shown. The data were from one representative experiment (n = 3).

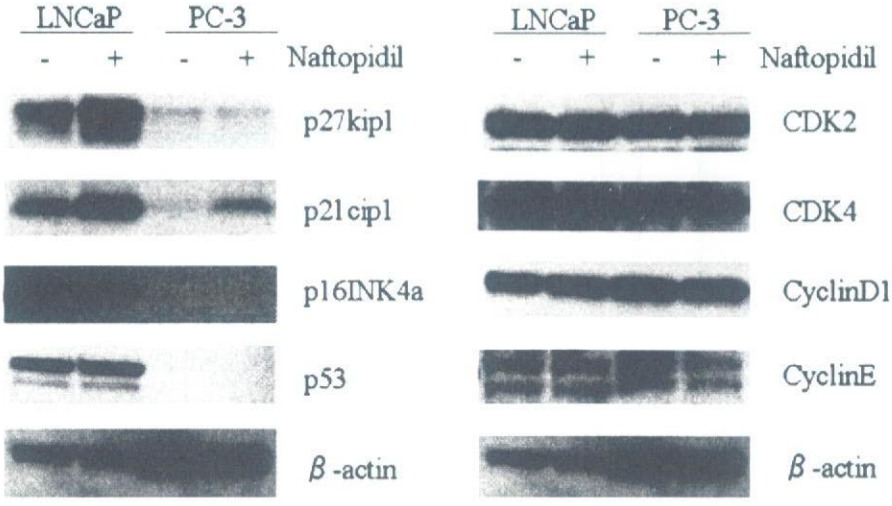

FIGURE 3 – Expression of positive and negative cell cycle regulators after naftopidil treatment. The cells were treated with naftopidil or vehicle for 24 hr. Cells lysates (30 μg) were subjected to western blot analysis with the indicated antibodies.

positive and negative G1/S checkpoint modulators in LNCaP and PC-3 cells by western blot analysis with specific antibodies (Fig. 3). In LNCaP, both $p27^{kip1}$ and $p21^{cip1}$ were increased by naftopidil treatment, whereas $p21^{cip1}$ was increased specifically in PC-3. Levels of p53, known to play an important role in cell-cycle regulation, did not change in LNCaP and was not detectable in PC-3. No appreciable change was observed in the protein levels of $p16^{INK4a}$, cyclin D1, cyclin E, CDK2 or CDK4.

Down-regulation of Akt signal in naftopidil-treated prostate cancer cells

As it has been reported that Akt might enhance cell cycle progression *via* diminishing the expression of $p27^{kip1}$ [26] and $p21^{cip1}$ [27] we evaluated the effect of naftopidil on Akt expression and activation. Akt expression levels were not changed; however, Akt activation (Ser-473 phosphorylation) was markedly decreased in PC-3 as compared to LNCaP (Fig. 4).

Effect of naftopidil on PSA secretion from LNCaP cells

To determine whether naftopidil affects PSA secretion from human prostate cancer cells, we investigated the change in PSA secretion from LNCaP cells. As shown in Figure 5, there was no significant difference in PSA secretion between 0.1 and 25 μM naftopidil treatments.

Inhibition of in vivo *tumor growth by naftopidil treatment*

As 1 tablet naftopidil is usually prescribed once daily in humans, we gave the drug to mice daily by oral administration for 28 days. As shown in Figure 6, control animals at day 28 had large tumors, of which relative volume was 3.91 ± 0.64 times at day 28. Treatments with 10 and 100 mg/kg/day naftopidil led to inhibition of relative tumor volume, which was 2.11 ± 0.49 and 1.79 ± 0.44 times, respectively, at day 28 (Fig. 6). A statistically significant reduction was observed in relative tumor volume between vehicle- and naftopidil-treated mice ($p < 0.05$), whereas no significant difference was noted between 10 and 100 mg/kg/day treatment with naftopidil.

Effects of naftopidil treatment on PC-3 tumors in vivo

No histopathological difference was observed between control and naftopidil-treated PC-3 tumors (data not shown). Result of immunohistochemical analysis was summarized in Table I. Naftopidil treatments significantly decreased Ki-67 index and increased the expression of $p21^{cip1}$ in PC-3 tumors. In naftopidil-treated group, the percentage of PC-3 cells undergoing apoptosis was not changed as compared to control group. MVD was significantly decreased in naftopidil-treated tumors, whereas no significant difference of MVD was observed between 10 and 100 mg/kg/day treatment with naftopidil.

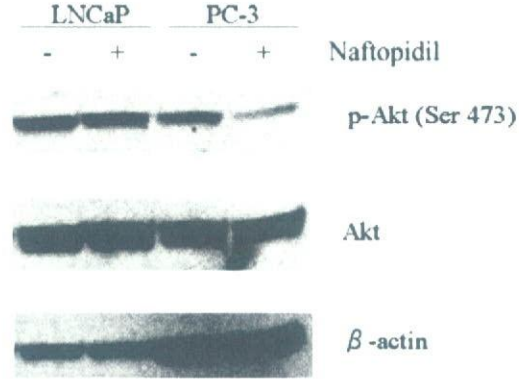

FIGURE 4 – Effect of naftopidil on Akt and phospho-Akt (Ser-473) protein levels. Total Akt and phospho-Akt protein levels were determined by immunoblot analysis using specific antibodies.

Discussion

In the present study, we have demonstrated that naftopidil has dose-dependent growth-inhibitory effects on human prostate cancer cell lines. The inhibition of cell growth observed in naftopidil-treated cells is apparently due to induction of G1 cell cycle arrest. Cell cycle inhibition could be an important target for cancer management, because of an association between deregulated cell cycle progression and cancer.[28,29] This antiproliferative effect indicates the potential value of naftopidil in therapy for prostate cancer. G1 cyclins (cyclin D and cyclin E) and cyclin-dependent kinases (CDK 2, CDK 4 and CDK 6) are critical positive-acting compo-

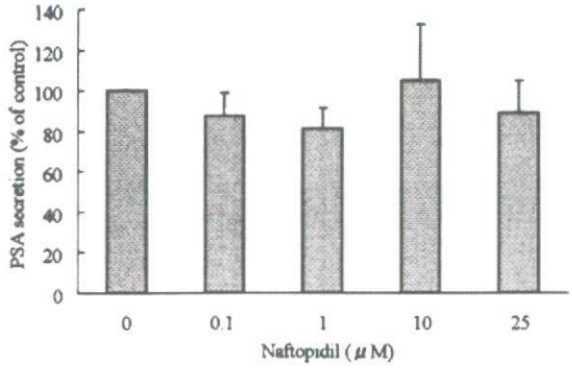

FIGURE 5 – Effect of naftopidil on PSA secretion from LNCaP. LNCaP cells were treated with various concentrations of naftopidil and secreted PSA in the culture medium were measured using an ELISA assay. The PSA concentrations were normalized to 10^6 alive cells and expressed as percentage over control.

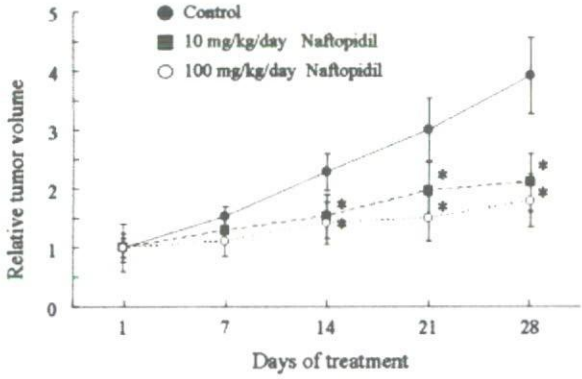

FIGURE 6 – Growth inhibition of PC-3 tumors treated with naftopidil. The volume of PC-3 tumors was measured weekly. Mice were given naftopidil (closed circle; vehicle control, closed square; 10 mg/kg/day, open circle; 100 mg/kg/day) once per day for 28 days. Results are expressed as the mean ± SD percentage of tumor volume compared to first treatment day ($n = 10$). Naftopidil-treated group resulted in a significant suppression of tumor growth compared to vehicle control ($*p < 0.05$).

nents for progression through the G1/S checkpoint of the cell cycle.[30,31] CDK inhibitors of the Kip1/Cip1 and INK4 families prevent activation of CDKs and their entry into S phase in mammalian cells. The Kip1/Cip1 family includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and the INK4 family includes $p16^{INK4a}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$.[32] Our study showed that expressions of $p27^{kip1}$ and $p21^{cip1}$ were significantly increased in LNCaP cells by naftopidil treatment. Interestingly, naftopidil treatment induced $p21^{cip1}$, but not $p27^{kip1}$, in PC-3. These results indicate that naftopidil-induced G1 cell cycle arrest is mainly associated with $p27^{kip1}$ increase in LNCaP and with $p21^{cip1}$ levels in LNCaP and PC-3, but not with up-regulation of $p16^{INK4a}$ and p53 or with down-regulation of G1 cyclins.

It is known that $p27^{kip1}$ is involved in controlled growth of the prostate gland because $p27^{kip1}$-deficient mice exhibit prostate hypertrophy.[33] In an immunohistochemical study, $p27^{kip1}$ was expressed in normal and benign prostate tissues, whereas this expression was down-regulated in neoplastic lesion.[34] Reduced $p27^{kip1}$ expression in neoplastic lesion could be associated with higher-grade disease and poor clinical outcomes.[34] Myers *et al.* demonstrated that androgen withdrawal by castration in male mice

TABLE I – SUMMARY OF PROLIFERATION OR APOPTOTIC INDEX AND MICROVESSEL DENSITY AMONG NAFTOPIDIL TREATED PC-3 XENOGRAFT

	Control ($n = 7$)	Naftopidil	
		10 mg/kg/day ($n = 7$)	100 mg/kg/day ($n = 7$)
Ki67 (%)	43.1 ± 11.4	29.9 ± 6.0*	14.7 ± 4.8*,**
$P21^{cip1}$ (%)	2.6 ± 0.4	6.8 ± 1.1*	15.7 ± 3.6**,***
Apoptosis (%)	6.0 ± 1.2	6.5 ± 0.8	6.1 ± 0.6
Microvessel density (number/field)	17.2 ± 1.9	11.7 ± 1.3*	11.5 ± 1.6*

Positive cells of Ki67, $p21^{cip1}$ and apoptosis were counted in 10 different areas at 400× magnification. Mouse-specific CD31 positive vessels with lumen were counted in 10 different areas at 200× magnification. Values represent the means ± SD.

$*p < 0.05$.

$**p < 0.01$: significantly different from the control value by Fisher's PLSD.

$***p < 0.05$: significantly different compared to 10 mg/kg/day group.

with androgen-sensitive prostate cancer, CWR22 xenograft, results in regression of the tumors and concomitant increase in $p27^{kip1}$ expression.[35] Re-establishment of testosterone levels in the castrated mice resulted in resumption of cellular proliferation and inhibition of $p27^{kip1}$ expression.[35] Therefore, induction of $p27^{kip1}$ could play an important role in the inhibition of androgen-sensitive prostate cancer cell growth.

In experimental studies, $p21^{cip1}$ suppressed proliferation of tumor cells *in vitro* and reduced tumorgenesis *in vivo*[36]; however, the relation between $p21^{cip1}$ expression and clinical prognosis remains controversial. In a radical prostatectomy study, $p21^{cip1}$ over-expression correlated with advanced stage and high risk for disease-free survival.[37] In contrast, Matsushima *et al.* reported that increased $p21^{cip1}$ expression is an independent predictor of favorable prognosis in 60 patients with prostate cancer, particularly in the half of whom had metastatic disease.[38] We observed induction of $p21^{cip1}$ in naftpidil-treated PC-3 tumors *in vivo* immunohistochemically. Moreover, $p21^{cip1}$ gene is inactivated in metastatic prostate cancer cell lines.[39] The induction of $p21^{cip1}$ but not $p27^{kip1}$ in naftopidil-treated PC-3 cells might suggest that $p21^{cip1}$ plays critical roles of tumor growth suppression in metastatic and androgen-insensitive prostate cancer cells. We also showed that Akt activation (Ser-473 phosphorylation) was markedly reduced in PC-3 as compared to LNCaP. In regard to the relation between Akt and $p21^{cip1}$, it was demonstrated that Akt pathway modulates $p21^{cip1}$ through phosphorylation at Thr145. This phosphorylation negated the inhibitory effect of cyclins/CDKs and resulted in the proliferation of cells.[40] In addition, the phosphorylated state of $p21^{cip1}$ was recognized by the proteasome and did cause its degradation.[27] Therefore, $p21^{cip1}$ expression after naftopidil treatment in PC-3 could be caused by this diminished Akt activity.

$p21^{cip1}$ and $p27^{kip1}$ are usually controlled by both p53-dependent and -independent mechanisms.[41–44] Since p53 expression was not changed in naftopidil-treated LNCaP cells, naftopidil-induced growth arrest in prostate cancer cells may be mediated through a p53-independent pathway. Mutations affecting p53 are frequently observed in advanced cancer[45]; therefore, the p53-independent action of naftopidil in cessation of cell proliferation may make it an attractive anti-cancer agent in the treatment of advanced prostate cancer.

α-1 Adrenoceptors are mainly expressed in the prostate gland.[14] In the present study, we also confirmed that human prostate cancer cell lines LNCaP and PC-3 express α-1 adrenoceptor subtypes. As results, α-1A subtype mRNA was highly expressed in LNCaP but not in PC-3. In contrast, α-1B subtype mRNA was detected in PC-3 but not in LNCaP. α-1D subtype mRNA was expressed in both LNCaP and PC-3 (data not shown). α-1 adrenoceptor stimulation induces cell proliferation and increases DNA synthesis in variety

of cell types[46,47]; however, α-1 adrenoceptor subtypes have differing effects on cell growth. Gonzalez-Cabera *et al.* demonstrated that α-1A adrenoceptor generally causes cell growth inhibition, whereas α-1B and 1D adrenoceptors have different effects depending on the treated cells.[48] As regard to the prostate cancer, Quaglia *et al.* demonstrated that α-1B and 1D adrenoceptors are expressed and norepinephrine have growth stimulation effect in PC-3.[49] We also demonstrated that PC-3 cells express α-1B and 1D adrenoceptors, whereas LNCaP cells express α-1A and 1D adrenoceptors. Therefore, growth-inhibitory effects of naftopidil could be yielded *via* antagonistic effect against α-1D adrenoceptor. However, Quaglia *et al.* confirmed new compounds showing high affinity to α-1D adrenoreceptor and demonstrated apoptotic effect of those agents on PC-3.[49] We attempted in our study to identify apoptosis after naftopidil treatment, but Hoechst 33258 staining did not identify apoptosis: DNA ladder formation and PARP cleavage were not observed in the naftopidil-treated cells (data not shown). Moreover, *in vivo* experiments, exchange of apoptotic index among control and naftopidil treatments were not observed. These results suggest that there is another mechanism by which naftopidil causes G1 cell cycle arrest in prostate cancer cells.

Whether or not men with LUTS are at increased risk of prostate cancer has not been determined.[50] Matsubara *et al.* demonstrated that, among Japanese men who attended a mass screening program, men with LUTS were not at higher risk than men without LUTS.[51] In male outpatients with LUTS, however, the incidence of prostate cancer was significantly higher than that among the mass-screened patients.[52] In addition, Fujikawa *et al.* reported that, because of increasing adoption of western diet in Japan, the frequency of latent prostate cancer increased from 20 to 30%, and the occurrence rates of moderately or poorly differentiated cancer in the period from 1980 to 1993 were 1.8 times as high as those in the period from 1966 to 1974.[53] Prescription of naftopidil for male outpatients with LUTS may have benefit of prostate cancer reduction in these patients with latent prostate cancer, who are particularly at high risk of clinical progression.

It is well known that finasteride, a 5α-reductase inhibitor, is a chemopreventive agent prescribed for BPH patients. The Prostate Cancer Prevention Trial showed that prostate cancer prevalence decreased by 24.8% in the finasteride-treated group; however, finasteride also seemed to increase the risk of high-grade prostate cancer.[54] Moreover, finasteride decreased PSA expression *in vitro*[55] and *in vivo*.[56] These results might produce a widespread resistance to the use of finasteride for preventing prostate cancer. PSA is a serine protease with a highly prostate-specific secretion. Clinically, serum PSA is the most sensitive and important biochemical marker available for monitoring the presence of prostate cancer and the response to the therapies.[57] For prostate cancer detection, a standard PSA cutoff of 4 ng/ml is recommended.[58] Pharmacological therapies for BPH that alter the serum PSA level confound monitoring for prostate cancer detection. In our study, we demonstrated that naftopidil did not affect the PSA secretion from androgen-sensitive LNCaP prostate cancer cells. The expression and secretion of PSA are mediated by androgen receptor (AR) at the transcriptional level.[59] Our results showed that naftopidil did not change PSA secretion from androgen-sensitive LNCaP cells, suggesting that activity of AR was not affected by naftopidil. In clinical aspect, no effect on PSA secretion by α-1 adrenoreceptor antagonisit, such as naftopidil, is advantageous for longitudinal monitoring of serum PSA in BPH patients.

Although LUTS is frequently seen in all urology units, few reports are available with data to establish the incidence of LUTS in HRPC patients. In addition to relieving LUTS, naftopidil might also have potential to improve the treatment outcomes of HRPC. Doxazosin is a well-investigated α-1 adrenoceptor antagonist with anti-tumor effect on prostate cancer cells[21,25,60-62]; however, it is uncertain whether prescription of doxazosin should be avoided in patients with heart failure: the Anti-Hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial study demonstrated that high-risk hypertensive patients taking doxazosin have doubled risk of developing heart failure.[63] In an autopsy study, latent prostate cancer correlated with coronary heart disease,[64] and moreover, cardiovascular disease had the strongest association with the cause of death in prostate cancer patients.[65] Although no explicit study regarding the influence of naftopidil on heart disease has been reported, selective α-1 adrenoceptor antagonists (for α-1A and 1D) could be suitable as treatments for LUTS in patients having prostate cancer complications.

In pharmacokinetics studies, the maximal therapeutic serum concentration of naftopidil ranged from 0.31 to 1.91 μM,[66] and that of doxazosin reached 0.27 μM.[67] Our experimental concentrations were not within the clinically-relevant therapeutic dose range, and were similar to those of doxazosin.[21]; However, a relation between treatment with quinazoline-based α-1 adrenoceptor antagonists and decrease in the relative risk of prostate cancer was observed in another study.[22] There also might be a possibility of growth-inhibitory effect of naftopidil on clinical early prostate cancer within the clinically-relevant therapeutic dose range.

In conclusion, naftopidil inhibited cell proliferation both *in vitro* and *in vivo*, and caused cell cycle arrest linked to the induction of $p27^{kip1}$ and $p21^{cip1}$ in LNCaP cells and $p21^{cip1}$ in PC-3 cells. To our knowledge, this is the first evidence for antiproliferative effects of naftopidil in prostate cancer cells. In the future, further clinical studies will be needed to ascertain the role of naftopidil as a chemopreventive or therapeutic agent against prostate cancer.

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Estrogen-responsive genes newly found to be modified by TCDD exposure in human cell lines and mouse systems

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) can induce estrogenic action or inhibit estrogen-induced effects in various tissues because of aryl hydrocarbon receptor (AhR)-estrogen receptor (ER) cross-talk. In order to identify the biomarkers of TCDD endocrine disruption, we screened estrogen-responsive genes modified by TCDD exposure using specific cDNA microarrays spotted with estrogen-responsive genes. MCF-7 human breast carcinoma cells and RL95-2 human endometrial carcinoma cells were exposed to TCDD, and an analysis of their gene expression revealed 32 genes exhibiting a significant change. The mRNA expression levels of 27 genes were subsequently verified using real-time RT-PCR. Among these genes, bioinformatic analyses indicated that insulin-like growth factor-binding protein 5 (*IGFBP5*) gene expression might be influenced by estrogen status. In our animal experiments, *IGFBP5* was also shown to be responsive to TCDD exposure in mouse fetuses *in utero*. These results suggest that TCDD affects the expression levels of a series of estrogen-responsive genes, and follow-up fetal studies in mice indicated that *IGFBP5* is useful as a biomarker of TCDD activity.

Keywords: Dioxin; Environmental estrogen; Estrogen receptor; IGFBP; Microarray analysis

1. Introduction

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits a wide spectrum of toxicities, including carcinogenicity, hepatotoxicity, and reproductive and developmental toxic responses (Birnbaum and Tuomisto, 2000; Yonemoto, 2000). These effects are thought to be mediated by the binding of TCDD to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor and a member of the basic helix-loop-helix per-arnt-sim family of proteins (Burbach et al., 1992; Whitlock, 1993). The liganded AhR translocates to the nucleus and heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer complex then binds to specific *cis*-acting enhancers known as xenobiotic or dioxin response elements (XREs or DREs). Upon binding to the XREs, the heterodimeric AhR-ARNT complex promotes the activation of a significant number of different genes (Denison et al., 1989; Hankinson, 1995). Hence, many toxic effects of TCDD are associated with altered gene expression.

Recently, TCDD has attracted a great deal of attention as an endocrine disruptor (Birnbaum, 1995). TCDD has now been shown to cause progressive endometriosis in the rhesus monkey (Rier et al., 1993), to suppress the development of the male reproductive system (Gray et al., 1997; Mably et al., 1992a,b; Ohsako et al., 2001), to induce sexual dimorphism in the rat brain (Zareba et al., 2002), and also to cause damage to the ovaries (Chaffin et al., 1996; Davis et al., 2000). Many important studies have demonstrated the existence of inhibitory AhR-estrogen receptor (ER) cross-talk in rodent uteri and mammary glands, as well as in human breast cancer cells. In human mammary cell lines and ER-positive human breast cancer cells, TCDD inhibits 17β-estradiol (E2)-dependent cell proliferation (Biegel and Safe, 1990) and prevents the secretion of E2-induced proteins, such as tissue plasminogen activator, cathepsin-D and pS2 (Gierthy et al., 1987). Significantly, none of these effects

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have been found in ER-negative breast cancer cells (Dohr et al., 1995).

In our previous work, we investigated AhR-ER cross-talk both *in vivo* and *in vitro*, and we showed that estrogen treatment enhances TCDD-induced cytochrome P450 1A1 (*CYP1A1*) expression in the livers of ovariectomized rats, suggesting that ER signaling can modulate AhR signaling (Sarkar et al., 2000). We have also shown in previous studies that ER-α acts as a positive modulator in the regulation of TCDD-inducible genes and is necessary for the transactivation of genes mediated through the XRE in both the human breast carcinoma cell line MCF-7 and human endometrial epithelial carcinoma cell line RL95-2 (Jana et al., 1999, 2000). There is substantial evidence that TCDD does not interact directly with the ER or the progesterone receptor (PR) (Romkes and Safe, 1988), so that the antiestrogenic effects of TCDD cannot be explained by direct interaction with these receptors, but must involve modulation of estrogen-induced gene transcription (Kharat and Saatcioglu, 1996). Recently, Ohtake et al. (2003) showed that the liganded AhR/ARNT heterodimer directly associated with unliganded ER, with the concomitant activation of the p300 coactivator, resulting in activation of the transcription of estrogen-responsive genes. They have also shown that the activation of AhR by dioxins impaired the expression of estrogen-responsive genes when E2 was present. It was suggested that ligand-activated AhR signaling varies depending on the estrogen-liganded status of the ERs. Based on those observations, it could also be speculated that the interaction between TCDD and ER signaling affects expression of a variety of genes.

Although it has now been reported that the interactions between estrogen and TCDD affect the gene expression or protein levels of cathepsin-D, pS2, PR, HSP27 and c-fos, these genes cannot explain the mechanisms of the estrogen-mimicking or inhibitory action of TCDD, and other novel biomarkers may hold the key to the effects of TCDD on estrogen-related development. In order to further shed light on the mechanisms of the interactions between estrogen and TCDD as an endocrine disruptor, a more comprehensive identification of the genes/pathways modulated by TCDD is needed.

Our previous study using cDNA microarrays of estrogen-responsive genes (EstrArray) to evaluate the estrogen activity of various chemicals has shown that TCDD displayed properties that were distinct from those of other environmental hormones such as bisphenol A, diethylstibestrol or genistein (Terasaka et al., 2004). EstrArray has been developed as a customized DNA microarray for the expression profiling of estrogen-responsive genes, which are assessed in order to detect the estrogen activity of environmental chemicals (Inoue et al., 2002).

In the present study, we identified genes displaying sensitivity to TCDD from a series of estrogen-responsive genes in the customized array. We used both human breast carcinoma cells (MCF-7) and human endometrial carcinoma cells (RL95-2), since breast cells and uterine endometrial cells are the principal targets of the endocrine-disrupting effects of TCDD. Furthermore, the expression levels of genes identified in these microarray screens were investigated in mouse fetuses exposed to TCDD *in utero*.

2. Materials and methods

2.1. Cell culture and treatment

The MCF-7 (human breast carcinoma) and RL95-2 (human endometrial carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells were grown in DMEM/Ham's F12 medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum. In our experiments, this growth medium was replaced with phenol red-free DMEM/Ham's F12 medium containing 10% charcoal-stripped FBS, and at 80% confluency the cells were treated with either 0.1, 1 or 10 nM TCDD (99.0% purity; 50 μg/ml DMSO solution, Cambridge Isotope Laboratories, Andover, MA) or with 10 nM estrogen (17β estradiol, Sigma–Aldrich, St. Louis, MO) in 0.1% DMSO for 24 h. The control cell cultures were treated with 0.1% DMSO only. All the culture media contained 100 units/ml penicillin and 100 μg/ml streptomycin, and each culture was maintained in a humidified 5% CO_2 atmosphere at 37 °C.

2.2. Animal use and treatment

C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan), and handled in accordance with the guidelines for animal welfare of the National Institute for Environmental Studies (NIES) of Japan. Breeding was carried out by co-housing one male and two females overnight. The plug day was designated as Day 0 of gestation (GD0). The mice in the control and dose groups (three dams in each group) were administered either a single oral dose of corn oil (0; vehicle) or 5 μg TCDD/kg, respectively, on GD12.5. On GD18.5, the dams were sacrificed using diethyl ether anesthesia, and 3 male and 3 female fetuses per dam were removed to enable RNA extraction from the liver, brain and calvaria.

2.3. cDNA microarray analysis

The mRNA for the microarray preparations was isolated using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, NJ), in accordance with the manufacturer's instructions. For cDNA labeling, 2 μg of mRNA from the control cells and the TCDD-treated cells were reverse-transcribed in the presence of Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia Biotech), respectively. The reverse transcription reactions were performed in a final volume of 20 μl, containing 2 μg of denatured mRNA with 0.5 μg each of the 6-mer and 9-mer random primers, 0.5 mM each of dATP, dGTP and dCTP, 0.2 mM dTTP, 2 μl of either Cy3 or Cy5 dUTP, 100 units of RNasin (Promega, Madison, WI), 10 mM DTT, and 200 units of Superscript II reverse transcriptase (Gibco BRL) in 1× buffer. The mixture was incubated at 42 °C for an initial 30-min period, and 200 units of Superscript II were then added. The mixture was then incubated for a further 30 min, after which the reactions were stopped. The residual RNA in each sample was then degraded with 5 μl of 0.5 M EDTA and 10 μl of 1N sodium hydroxide for 60 min at 65 °C. The solutions were neutralized with 25 μl of 1 M Tris–HCl (pH 7.5), and the resulting probes, labeled with either Cy3 or Cy5, were combined and purified using a Microcon-30 filter (Millipore, Bedford, MA). In these experiments, custom cDNA microarrays spotted with estrogen-responsive genes (EstrArray, Infogenes Co., Ltd., Tsukuba, Japan) were used (Inoue et al., 2002). The fluorescent probe pair was hybridized onto the cDNA-printed slide glasses in 20 μl of hybridization buffer (5× SSC plus 0.5% SDS) under a coverglass for 12 h at 65 °C. The hybridized slides were washed three times with 2× SSC and 0.2% SDS for 5 min at room temperature, three times with 0.2× SSC and 0.2% SDS for 5 min at room temperature, three times with 0.2× SSC and 0.2% SDS for 5 min at 60 °C, three times with 0.2× SSC and 0.2% SDS for 5 min at room temperature, then rinsed four times with 0.2× SSC at room temperature. Both scanning and data analyses were performed in this procedure, as in the previous study (Terasaka et al., 2004). For each spot, the signal intensity ratio of Cy3 to Cy5 (Cy3/Cy5) was calculated and $\log_2$-transformed. Each $\log_2$(Cy3/Cy5) value was normalized to 27 internal control genes *ACTB*, *ACTN1*, *CPEB2*, *FLJ12748*, *FUSIP1*, *G0S2*, *G6PD*, *GCLM*, *GTF2H2*, *HNRPK*, *IL6ST*, *KANK*, *KIAA0349*, *KRT6E*, *LOC129401*, *NAV1*, *NMA*, *NPM1*, *PAK4*, *PRKCD*, *RPL35*, *SDR1*, *SLC25A16*, *SLC29A2*, *SOCS2*, *TNFRSF7*, and *ZNF147* that were included on the chip, and the duplicated $\log_2$(Cy3/Cy5) values for each gene

were averaged. The gene expression levels were considered to have been genuinely altered by the treatment when the averaged value of $\log_2$(Cy3/Cy5) was either greater or less than 1.

2.4. Quantitative real-time RT-PCR

Total RNA for real-time RT-PCR was isolated using ISOGEN (Wako, Osaka, Japan) and RNeasy (QIAGEN, Valencia, CA), in accordance with the manufacturer's instructions. The integrity of the RNA samples was verified by spectrophotometric analysis and formaldehyde agarose-gel electrophoresis. Two and a half μg of each total RNA sample were reverse-transcribed in a 50-μl reaction mixture containing 5 mM of each dNTP, 62.5 units of reverse transcriptase enzyme (MultiScribe, Applied Biosystems, Foster City, CA), 2.5 μM oligo(dT) primers, 1× RT buffer, and 5.5 mM $MgCl_2$. The reaction mixtures were incubated at 25 °C for 10 min, 48 °C for 40 min, and finally 95 °C for 5 min. The resulting cDNA (1 μl) was amplified in triplicate using gene-specific primers and TaqMan probes (Sawady Technology, Tokyo, Japan) using TaqMan 1000 RXN Gold with Buffer A Pack (Applied Biosystems) or only the primers from the SYBR Green PCR Core Reagents (Applied Biosystems), in accordance with the manufacturer's instructions. The amplification reaction was performed on an ABI PRISM 7000 Sequence Detector (Applied Biosystems) under the following cycling conditions: 95 °C for 15 min, and 50 cycles of 95 °C for 15 s and 60 °C for 60 s. The gene expression levels were calculated based on the threshold cycle (Ct) using Sequence Detection System Software (Applied Biosystems) and normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The assays were performed in triplicate, and the statistical comparisons were carried out by ANOVA followed by the Bonferroni/Dunn test implemented on StatView 5.0 (SAS Institute Inc., Cary, NC). The specificity of each of the PCR products was confirmed by sequencing with gene-specific primers. Cycle sequencing reactions with Taq DNA polymerase were performed with fluorescence-labeled dideoxynucleotides using BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems), in accordance with the manufacturer's instructions. Sequence database searches were performed with BLAST sequence comparison programs at the National Center for Biotechnology Information.

2.5. Bioinformatic and statistical analysis

Clustering for gene expression was conducted in this study using GeneSpring 7 (Agilent Technologies, Palo Alto, CA). Gene expression data obtained by quantitative RT-PCR were mapped to biological networks using Ingenuity Pathway Analysis (Ingenuity Systems, Mountain View, CA), which is a tool for finding pathways based on information selected from published articles. The expression values at 10 nM TCDD verified by quantitative RT-PCR were used for this analysis. Insulin-like growth factor binding protein 5 (*IGFBP5*) expression in human and mouse tissues was obtained from the public database Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/). In the animal studies, deviations in the expression levels of genes relative to control levels were assessed by Student's *t*-test implemented on StatView 5.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Identification of estrogen-responsive genes modified by TCDD in human carcinoma cells

The microarray spots that we utilized in this study consisted of 203 cDNAs corresponding to 165 different estrogen-responsive genes selected as reported previously (Inoue et al., 2002; Terasaka et al., 2004). The gene expression profiles displayed a wide variation within the TCDD treatment range of 0.1–10 nM in both cell types. Hierarchical clustering analysis of 198 cDNA spots revealed the differences in expression profiles between MCF-7 and RL95-2 cells at doses of TCDD from

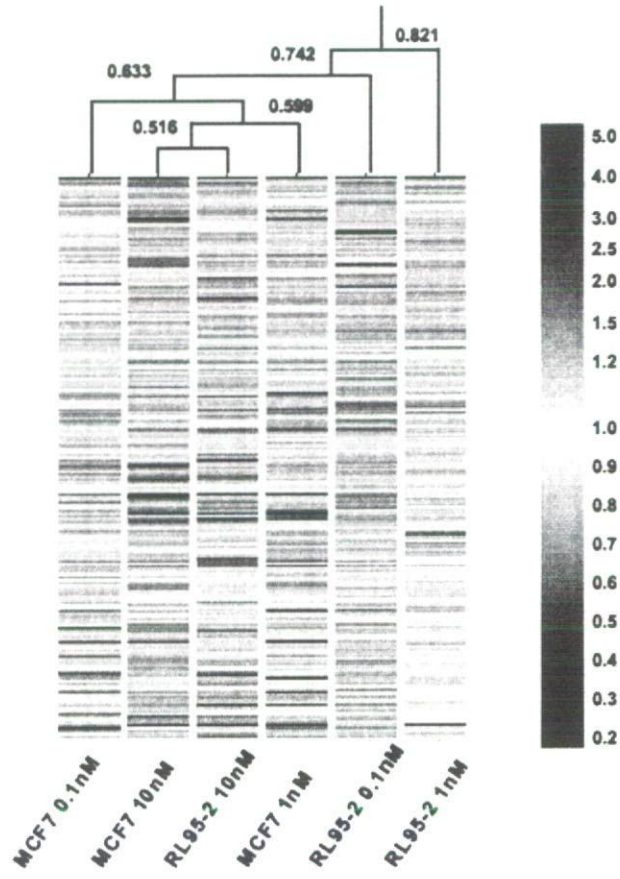

Fig. 1. Hierarchical clustering of estrogen-responsive genes, induced by TCDD, in MCF-7 and RL95-2 cells. Each bar represents the expression profile of each microarray. Bars were lined in the order 1, 4, 5, 6, 3 and 2 shown here, based on the results of the clustering analysis. The coloured bar indicates values of the ratio of the gene expression to control. Each value on the tree shoulders indicates the distance between two array profiles. The distance *D* represents 1 - correlation coefficient.

0.1 to 10 nM (Fig. 1). At 10 nM TCDD, the expression profiles between the two cell lines corresponded closely (indicated by MCF-7 10 nM and RL95-2 10 nM in Fig. 1), but they did not correspond closely at doses of 1 or 0.1 nM TCDD. The results suggested that the mechanisms of transcriptional activity at a high dose of TCDD might be different from those at a low one. Thirty-two genes were detected to have undergone change due to TCDD treatment in both cell lines (Table 1). In addition to these 32 genes, the *AHR* and *CYP1A1* genes that were used as positive controls in our screen were also induced by TCDD.

The number of genes responsive to TCDD exposure was found to increase in a dose-dependent manner (Fig. 2A). At the lowest TCDD dose (0.1 nM), some genes were found to be induced in the MCF-7 cells but not in the RL95-2 cells. Venn diagrams of the cell/concentration distribution of genes significantly responsive to TCDD are shown in Fig. 2B and C. Nineteen genes in the MCF-7 cells and 22 genes in the RL95-2 cells, including *AHR* and *CYP1A1*, were significantly altered by TCDD at all concentrations in the examined range.

Table 1
Estrogen-responsive genes modified by 0.1, 1 or 10 nM TCDD in either MCF or RL95-2 cells, as determined by EstrArray

Name	Accession No.	Description
AhR	L19872	Human AH-receptor mRNA, complete cds
APM2	BC004471	Adipose-specific 2, clone MGC: 10343, mRNA complete
BCL2L 11	AA629308	BCL2-like 11 (apoptosis facilitator)
BSN	Y18448	Bassoon (BSN) gene, exon 2 and joined CDS, partial
CDH18	U59325	Cadherin-14 mRNA, complete cds; cadherin 18, type 2
CPEB2	NM_182646	Cytoplasmic polyadenylation element-binding protein 2, clone: QnpA-21739
CPT1A	L39211	Carnitine palmitoyltransferase 1A, liver
CTNND2	NM_001332	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)
DHCR24	D13643	The KIAA0018 gene, 24-dehydrocholesterol reductase
ENPP2	L46720	Autotaxin-t(atx-t)gene, complete code.
ERBB2	NM_004448	Erythroblastic leukemia viral oncogene homolog 2; avian neuro/glioblastoma-derived oncogene homolog
MAP6D1	NM_024871	MAP6 domain containing 1 (*MAP6D1*), mRNA. Hypothetical protein FLJ12748 (AK0022810), clone NT2RP2001137
FUT8	Y17977	Fucosyltransferase 8 (alpha (1,6) fucosyltransferase)
GCLM	L35546	Gamma-glutamylcysteine synthetase light subunit mRNA, complete cds
GOS2	M72855	GOS2 gene, 5′ flank and cds; putative lymphocyte G_0/G_1 switch gene
CXCL12	NM_199168	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), transcript variant 1, mRNA. *CXCL12* is the official name of intercrine-alpha (hIRH, U19495)
IGFBP5	L27560	Insulin-like growth factor binding protein 5 (*IGFBP5*) mRNA
IGF1R	X04434	Insulin-like growth factor I receptor
ITPK1	U51335	Inositol 1,3,4-trisphosphate 5/6-kinase mRNA, complete cds
KIAA0373	AB002371	The *KIAA0373* gene, partial cds
MAN1A1	NM_005907	Mannosidase, alpha, class 1A, member 1
MAP4K5	AL118556	BAC R-406H23oq; mitogen-activated protein kinase 5 (*MAP4K5*)
ME1	U43944	Human breast cancer cytosolic NADP(+)-dependent malic enzyme mRNA, partial cds
NAV1	AB032977	mRNA from KIAA 1151 protein, partial. NAV1: neuron navigator 1
NRIP	X84373	Nuclear receptor interacting protein 1
PAC 204E5	Z98941	Human DNA sequence from *PAC 204E5* on chromosome 12. ATP ligand gated ion channel
PCYT1A	AI767533	Phosphate cytidylyl transferase 1, choline, alpha isoform; cDNA FLJ26328 fis, clone HRT01493
PR	X51730	Human mRNA and promoter DNA for progesterone receptor
RCN1	D42073	Reticulocalbin 1, EF-hand calcium-binding domain
SLC7A5	M80244	Human solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
TFIIH	AI239815	General transcription factor IIH, polypeptide 2 (44 kDa subunit)
TGFA	NM_003236	Homo sapiens transforming growth factor, alpha (TGFA), mRNA
VEGF	AF024710	Homo sapiens vascular endothelial growth factor (VEGF) mRNA, 3′UTR
CYP1A1[a]	K03191	Human cytochrome P-1-450 (TCDD-inducible) mRNA, complete cds

[a] Genes known to be induced by TCDD, used as positive controls in MCF-7 cells (Dohr et al., 1995; Jana et al., 1999). These genes are in all groups in Fig. 1B and 1C.

The expression levels of the estrogen-responsive genes *VEGF*, *MAN1A1*, *PCYT1A*, *ENPP2*, *GOS2* and *MAP4K5*, commonly altered in both cell lines, were also found to be altered by TCDD exposure. We anticipated that in different cell types the transcriptional mechanism, including activators and repressors, would differ and result in dissimilar TCDD-induced gene expression profiles.

3.2. Verification of gene expression changes in human carcinoma cells by real-time RT-PCR

To quantitatively verify the changes in gene expression that were observed in our cDNA microarray experiments, we determined by quantitative RT-PCR the expression levels of the 27 genes responsive to TCDD (Table 2). These included *CYP1A1*, the expression of which was used as a positive control. We were able to successfully detect the expression levels of 25 genes in the MCF-7 cells and 15 genes in the RL95-2 cells by real-time RT-PCR, as shown in Table 2. Thirteen genes, including *CYP1A1*, showed altered expression levels following TCDD treatment in both the MCF-7 and the RL95-2 cells. These genes may serve as biomarkers of TCDD exposure, since they responded to TCDD treatment in two different cell types. The typical dose responses to TCDD for six genes listed in Table 2 are represented as graphs in Fig. 3. MCF-7 cells were found to be more responsive to TCDD than RL95-2 cells. Expression of *SLC7A5* and *CYP1A1* was dose-dependently increased by TCDD exposure in MCF-7 cells, although the *SLC7A5* expression level was much lower than that of *CYP1A1*. Expression of *CDH18* and *CPEB2* was decreased dose-dependently in MCF-7 cells, while that of *IGFBP5* was decreased by exposure to varying concentrations of TCDD, but not dose-dependently.

To compare estrogen-responsiveness, 11 of the 26 genes that had been verified as being TCDD-responsive by quantitative RT-PCR were evaluated in MCF-7 and RL95-2 cells exposed

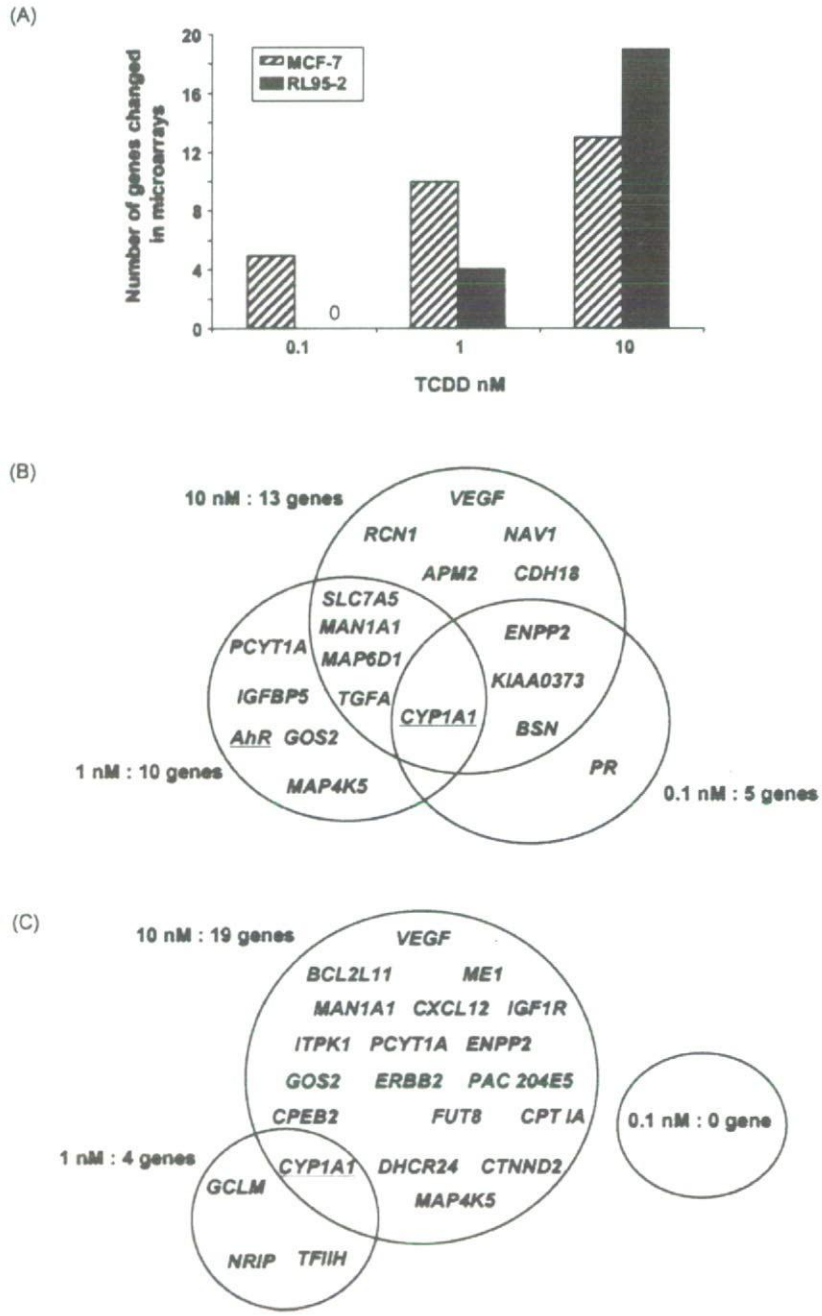

Fig. 2. Differential expression of the estrogen-responsive genes, induced by TCDD, by dose level and cell type. (A) An increase in the combined number of genes shows elevated expression levels in response to increased TCDD doses. (B) Venn diagrams of specific genes that respond to TCDD exposure in MCF-7 cells. (C) Venn diagram of specific genes that respond to TCDD exposure in RL95-2 cells. *AhR* and *CYP1A1* have been previously characterized as TCDD-responsive genes and were used as positive controls in the present study.

to estrogen (Table 3). *CPEB2*, *IGFBP5*, *ERBB2*, *CTNND2* and *CYP1A1* responded two-fold less than the control to estrogen exposure at all of the doses of 0.1, 1 or 10 nM in MCF-7 cells. These inhibitory trends were observed in the results from exposure to TCDD (Table 2). In RL95-2 cells, expression of *CPEB2* and *CYP1A1* was decreased by estrogen exposure. In contrast, expression of *CXCL12* and *SLC7A5* in MCF-7 cells, and *IGFBP5* in RL95-2 cells, was increased.

3.3. Bioinformatic analysis of verified expression data for MCF-7 and RL95-2 cells

The values of the verified expressions of nine genes (*CDH18*, *CTNND2*, *ERBB2*, *CXCL12*, *IGF1R*, *IGFBP5*, *TGFA*, *NRIP1* and *CYP1A1*) which were altered by TCDD in the MCF-7 and RL95-2 cells were used for pathway analysis, which is a bioinformatics' tool for determining the relationship between the