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Down-regulation of Skp2 is Correlated with p27-associated Cell Cycle Arrest Induced by Phenylacetate in Human Prostate Cancer Cells

TAKUJI SHIBAHARA, TAKEHISA ONISHI, OMAR E. FRANCO, KIMINOBU ARIMA and YOSHIKI SUGIMURA

Department of Urology, Mie University Facutly of Medicine, Tsu, Mie, Japan

Abstract. *We have demonstrated that phenylacetate(PA)-induced cell cycle arrest in human prostate cancer is mediated by increase of p27. In this study, we further investigated the mechanism of PA-induced p27 expression in prostate cancer cells (LNCaP, androgen-independent LNCaP [AIDL] and PC-3). A striking decrease in Skp2 mRNA and protein expression and reciprocal increase in p27 protein level were observed in three PA-treated prostate cancer cells. Interestingly, reduction of phospho-Akt and up-regulation of p27 mRNA levels were observed only in PC-3 cells. No significant differences were found in phospho-Akt and p27 mRNA levels in LNCaP and AIDL. In vitro ubiquitination assay showed a decreased p27 ubquitination in PA-treated prostate cancer cells. Our results suggest that PA attenuated Skp2 expression, thereby inhibiting ubiquitination and promoting p27 accumulation in all three prostate cancer cell lines. Therapeutic strategies designed to reduce Skp2 may clinically play an important role in the treatment of both androgen-sensitive and hormone-refractory prostate cancer.*

Androgen ablation therapy is the standard for advanced prostate cancer (1). This therapy is initially very effective, however, most advanced, especially metastatic cancers, tend to be resistant to androgen deprivation strategies during continuous hormone depletion therapy. Once in the state of androgen independence, this therapy is no longer effective (2), and no other therapy, including cytotoxic chemotherapy, is available at present. Therefore, novel therapies for hormone-refractory prostate cancer are warranted.

Correspondence to: Takehisa Onishi, M.D., Department of Urology, Mie University Faculty of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel: 81-59-232-1111, ext. 6456, Fax: 81-59-231-5203, e-mail: takehisa@clin.medic.mie-u.ac.jp

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We noted that phenylacetate (PA), a physiological product of phenylalanine metabolism, is present at micromolar levels in human plasma (3) and is conjugated to glutamine in the liver by phenylacetyl coenzyme A to form phenylacetylglutamine. Based on this feature, it has been used safely to treat children with inborn errors of urea synthesis and also in patients with hyperammonemia (4, 5). PA has been shown to be a nontoxic differentiation activator and a new class of tumor growth-inhibitory compounds. It has been demonstrated that treatment with millimolar concentrations of PA resulted in cytostasis, growth inhibition and differentiation in various hematological and solid neoplasms, including prostate cancer (6-12).

Our previous study showed that PA induced cell cycle arrest at G1-S transition, and reduced phosphorylation of the retinoblastoma protein (pRb) and cyclin-dependent kinase 2 (CDK2) activity by increasing p27 protein levels in human prostate cancer cells (13). The results implicate that PA-induced growth inhibition is mainly *via* regulation of p27 status in prostate cancer cells. p27 is a member of the Kip1/Cip1 family of cyclin-dependent kinase inhibitors (CDKIs), which negatively regulates cyclin-CDK complexes at the G1-S transition, thus inhibiting entry into the S-phase of the cell cycle. It has been shown that low levels of p27 are correlated with tumor grade, recurrence rate and prognosis in several cancers (14-18).

The abundance of p27 is regulated by a post-translational mechanism, mainly through the ubiquitin-mediated protein degradation system (19). This ubiquitination is performed by S-phase kinase-associated protein 2 (Skp2), which is a member of the F-box family (20). Binding of p27 to Skp2 requires its phosphorylation at threonine residue 187 by cyclin-CDK complexes, and this binding is greatly increased by the CDK subunit 1 (Cks1) (21). Previous studies also demonstrated that the transcription of p27 is activated by AFX (22), which is negatively regulated by Akt (23-26). Akt is a critical regulator of cell proliferation and survival and induces cell invasiveness

and angiogenesis (27). Overexpression of Akt isoforms has been reported in several cancers (28-30). In androgen-independent LNCaP cells, Akt activity was increased and diminished p27 expression was seen, compared to the androgen-dependent LNCaP (31).

The present study was undertaken to investigate the mechanisms by which PA induces p27 expression in human prostate cancer cells. We noted that PA modulates p27 expression mainly by post-translational regulation through Skp2 and partly by AKT-mediated transcriptional regulation.

Materials and Methods

Materials. Phenylacetate (PA) was purchased from Wako LIFE Science (Osaka, Japan). Antibody against p27 was obtained from Transduction Laboratories (Lexington, KY, USA), against Skp2 from Zymed Laboratories (San Francisco, CA, USA) and against Akt and phospho-Akt from New England Biolabs (Beverly, MA, USA). [α-^{32}P]dCTP was purchased from Amersham Biosciences (Piscataway, NJ, USA).

Cell culture. The human prostate cancer cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI-1640 (LNCaP) or DMEM/F12 (PC-3) medium supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μ/ml), in a water-jacketed incubator with a humidified atmosphere (5%CO_2, 95% air) at 37˚C. AIDL (androgen-independent LNCaP cells), which can grow very well in the absence of androgen, was induced by being maintained in phenol red-free RPMI-1640 medium with 2% charcoal-stripped FBS for over 2 years, as described previously (13). Experimental cultures were grown to 60-70% confluency and then treated with 5 mM PA or vehicle (ethanol) by replacing the medium supplemented with 10% FBS and either PA or ethanol. The amount of ethanol added as the vehicle never exceeded 0.1% of the total volume.

Western blot analysis. Cells were rinsed twice with ice-cold PBS and lysed in 1 ml of NP-40 lysis buffer: 50 mM Tris-HCl[pH 7.4], 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 2 mM sodium vanadate, 0.5% sodium deoxycholate 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin and 0.1% NP-40. Protein concentrations were measured at least twice by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). For Western blot analysis, protein samples were boiled for 5 minutes in Laemmli buffer and separated on 6-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then transferred to nitrocellulose membrane (Hybond ECL, Amersham Biosciences) by a semi-dry transfer method. The transferred membranes were soaked in Ponseau-S solution (Sigma, St. Louis, MO, USA) to check for comparable amounts of proteins loaded and the homogeneity of transfer. After blocking with TBST-MILK (Tris-buffered saline[pH7.4], 0.1% tween 20, 5% low fat milk), the membrane for p27 was incubated with primary antibody (1:2500) for 1-2 hours in TBST-MILK and membranes for Akt and Skp2 (1: 1000) in TBST-BSA (TBST containing 5% BSA). Then the membranes were incubated with secondary antibody conjugated with HRP for 1 hour, before developing with the enhanced chemiluminescence (ECL) detection system (PIERCE, Rockford, USA).

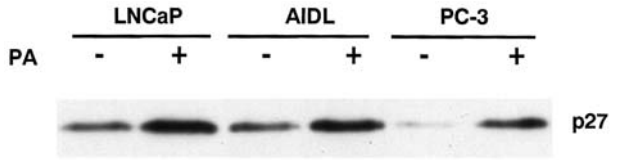

Figure 1. *Effect of PA on p27 protein expression in LNCaP, AIDL and PC-3 cells. Cells were treated with 5mM PA for 24 hours. Fifty μg of total cell lysates were subjected to immunoblot analysis with p27 antibody.*

Northern blot analysis. Total RNA was extracted from growing cells using ISOGEN (Waco Pure Chemical Industries , Osaka, Japan). Electrophoresis was performed on 1.2% agarose-formaldehyde gel (sample 15 μg) and the gels were transferred to Hybond NX nylon membranes (Amersham Biosciences) in 20XSSC (3 M NaCl, 8 mM NaOH, 2 mM sarkosy) and were fixed by UV crosslinker. DNA fragments for p27 and Skp2 were amplified by PCR as previously described (29), and radiolabelled using Prime-a-Gene Labeling System (Promega, Madison, WI, USA). Membranes were prehybridized and hybridized with [α-^{32}P]dCTP-labelled probes in Hybridization Solution (Nacalai Tesque, Kyoto, Japan). After hybridization, the membranes were washed in 2XSSC at 68˚C twice and 1XSSC at 68˚C twice. Autoradiography was performed using Amersham ECL X-ray film at –70˚C with an intensifying screen.

Real-time PCR. Total RNA was extracted as described above. After reverse-transcription, Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA, USA). The reaction mix contained cDNA template, PCR Master Mix (PE Applied Biosystems) and Assays-on-demand™ Gene Expression Products (PE Applied Biosystems) in total 50 μl for mRNA analysis of p27, Skp2 and 18S. For Cks1 mRNA analysis, primers were as follows: 5'-GGTCCATTATATGATCCATGAACCG-3', 3'-GAAAAGTCGGAGTTCGAAATGTGT-5', and probe was FAM-TTCCGGCACCCACTACCCGAGAA-TAMRA.

Ubiquitination assay. We used the Ubiquitinated Protein Enrichment Kit obtained from Calbiochem(La Jolla, CA, USA & Canada). Forty μl of polyubiquitin affinity beads were added to 2 mg of cell lysate and lysis buffer(50 mM HEPES[pH7.5], 5 mM EDTA, 150 mM NaCl and 1% Triton X-100) with protease inhibitor cocktail and 10 mM N-ethylmaleimide was added to a total volume of 1 ml, followed by incubation at 4˚C for 3 hours. After centrifugation for 5 seconds at 4˚C, the supernatant was removed and washed in 1 ml lysis buffer. The beads were suspended in 2X gel loading buffer(250 mM Tris-HCl[pH6.8], 4% SDS, 10% β-mercaptoethanol, 20% glycerol, bromophenol blue), and boiled for 5 minutes. After centrifugation for 1 minute at 13,700 xg, the lysates were separated on 8% SDS-polyacrylamide gel electrophoresis. Then the gel was transferred to Hybond ECL nylon membranes (Amersham Biosciences). After washing, blocking was performed for 1 hour. The membrane was incubated with primary antibody (p27, 1:1000) for 1-2 hours in blocking buffer and then the membranes were incubated with secondary antibody conjugated with HRP (IgG mouse, 1: 2500). Developing with ECL advance (Amersham Biosciences) autoradiography was performed. All experiments were performed in triplicate unless otherwise specified.

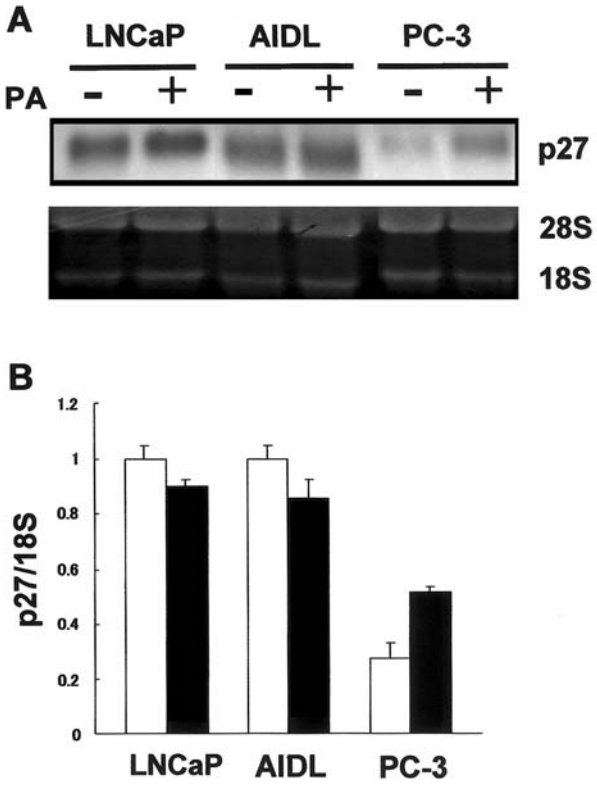

Figure 2. *Effect of PA on p27 mRNA expression. Total cellular RNA was extracted from LNCaP, AIDL and PC-3 cells after PA treatment for 24 hours. (A) Fifty μg of total RNAs were used for Northern blot analysis. (B) Expression of p27 mRNA was analyzed using quantitative real-time RT-PCR. Fold increase of mRNA in PA-treated cells were calculated comparing with eukaryotic 18S as an internal standard. About a two-fold increase was observed in PC-3 cells, although a significant increase was not demonstrated in LNCaP and AIDL cells. Open squares show control cells, and closed squares indicate PA-treated cells.*

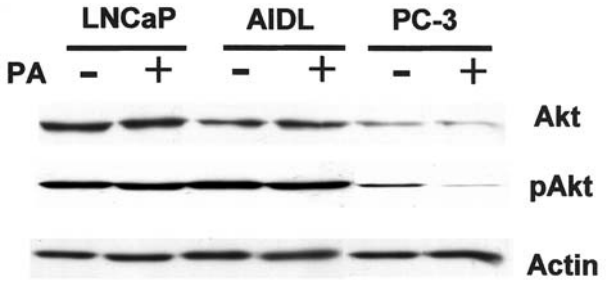

Figure 3. *Effect of PA on Akt and phospho-Akt (Ser-473) protein levels following PA treatment. Akt and p-Akt protein levels were determined by immunoblot analysis with Akt and phospho-Akt antibodies (using 50μg of cell lysates).*

Results

Effect of phenylacetate on p27 in prostate cancer cells. It is well known that PA induces a perturbation of the cell cycle progression, especially G1 arrest. Our previous report showed that PA concentrations in the range of 4-6 mM have antiproliferative effects in prostate cancer cells and that p27 is a key target in this process (13). We used the well-known LNCaP (androgen-dependent), PC-3 (androgen-independent) and an androgen-independent LNCaP (AIDL) cell line, which was isolated in our laboratory as described previously (13). In this study, cells were cultured in serum containing medium with 5 mM concentrations of PA for 24 hours, and proteins were extracted from these cells as described in Materials and Methods. We first examined the protein levels of p27. Western blot analysis showed that p27 protein levels were increased in PA-treated cells. Interestingly, p27 basal expression in PC-3 cells was lower than LNCaP and AIDL, suggesting that a more malignant state is associated with a lower level of p27 (Figure 1).

Next, to determine whether the increase in p27 was regulated transcriptionally, Northern blot analysis was performed. p27 expression did not change significantly in LNCaP and AIDL, however PC-3 cells exhibited very low basal levels of p27 mRNA, which was greatly elevated after PA addition (Figure 2A). The change of p27 mRNA expression was also analyzed by real-time RT-PCR. PC-3-treated cells demonstrated an increase of approximately 2-fold over control cells. The same as the results of Northern blot, LNCaP and AIDL cells did not show any remarkable changes (Figure 2B). These data suggest that PA-induced p27 expression might be regulated at the translational level in LNCaP and AIDL.

Akt activity in PA-treated prostate cancer cells. It has been reported that Akt may enhance cell cycle progression by diminishing expression of p27 (31). To assess the effect of PA on Akt expression and activation, total cell lysates from PA-treated cells were analyzed by Western blot. Although Akt expression levels were unchanged (Figure 3), Akt activation (Ser-473 phosphorylation) was markedly decreased in PC-3 cells compared to LNCaP and AIDL. The down-regulation of AKT activity (measured by Ser-473 phosphorylation) by PA could be one of the mechanisms of diminishing p27 expression in PC-3 cells. These data suggest that some mechanism other than the Akt pathway may be implicated in p27-regulation by PA in LNCaP and AIDL.

PA treatment down-regulates Skp2 expression in prostate cancer cells. Recent evidence has strongly suggested that the intracellular concentration of p27 is predominantly regulated by Skp2, an F-box protein, through the ubiquitin-proteasome proteolityc pathway (19, 20). Thus, to further characterize the involvement of this pathway in PA-treated

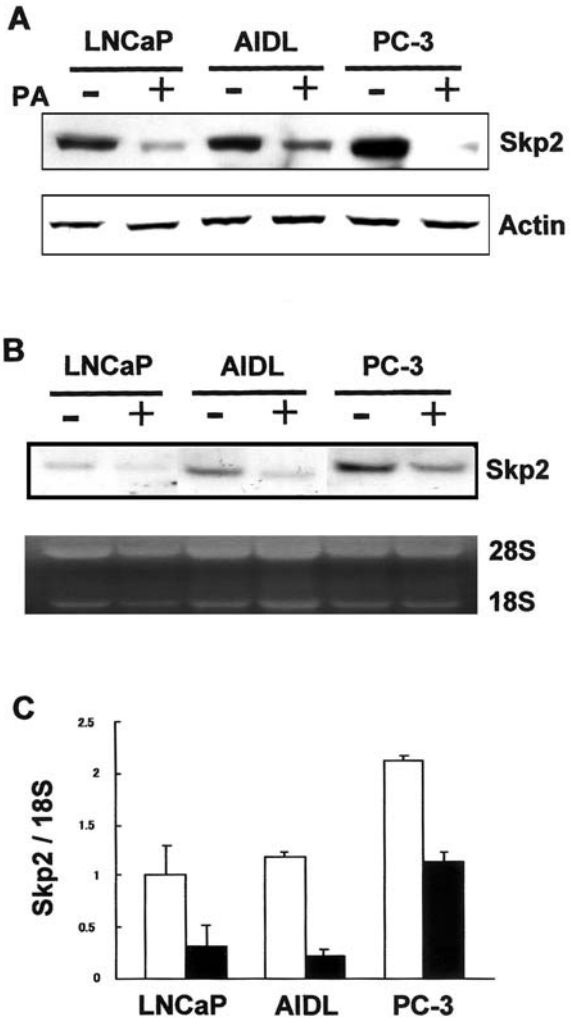

Figure 4. *Expressions of Skp2 protein and mRNA levels after PA treatment. (A) After 5 mM PA treatment, protein levels were examined by immunoblot analysis with Skp2 antibody. (B) Northern blot analysis showed a substantial decrease of Skp2 mRNAs in all three PA-treated cells. (C) Skp2 mRNA levels were determined using real-time RT-PCR. Compared with eukaryotic 18S mRNA level, each mRNA level of control and PA-treated cells was estimated.*

cells, we examined the expression of Skp2 by immunoblot analysis. Basal Skp2 expression was higher in PC-3 cells relative to LNCaP and AIDL cell lines. Skp2 expression decreased in PA-treated cells after 24 hours. This decrease was more pronounced in PC-3 cells (Figure 4A). Skp2 mRNA levels were also assessed by Northern blot and real-time PCR. Northern blot analysis showed a drastical decrease in Skp2 mRNA (Figure 4B). Quantitation by real-time PCR also showed a diminished expression, about 80% in LNCaP and AIDL cells, and more than 40% in PC-3 cells (Figure 4C). Evidence that Cks1 is essential for ubiquitin ligation of p27 and that it greatly enhanced the binding of

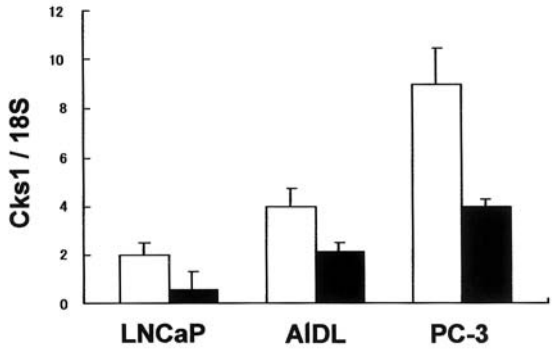

Figure 5. *mRNA expressions of Cks1 in PA-treated and untreated prostate cancer cells. Real-time RT-PCR analysis shows a clear decrease after 5 mM PA treatment (compared with eukaryotic 18S mRNA level). Open squares show control cells, and closed squares indicate PA-treated cells.*

T187-phosphorylated p27 has been reported (21). To investigate the role of Cks1 in these effects, real-time PCR was undertaken. Cks1 mRNA expression was higher in PC-3 cells than LNCaP and AIDL cells. A decreased Cks1 expression was noted after PA addition in all the cell lines. This decreased expression was in the range of approximately 50% to 75% being higher for LNCaP and lower for AIDL and PC-3 cells (Figure 5). These data suggest that Skp2 and Cks1 expression are modulated by PA and might be involved in p27 regulation.

PA-induced p27 up-regulation correlates with decreased p27 ubiquitination. Real-time PCR revealed that PA-induced Skp2 down-regulation at the protein level mirrors changes in the mRNA state in the studied cell lines. In contrast, p27 mRNA levels were up-regulated only in PC-3 cells, but were unchanged in LNCaP and AIDL cells. These results indicate that the effect of PA on p27 mRNA levels could not fully account for the accumulation of p27 protein, especially in the androgen-independent/sensitive AIDL and its parental androgen-dependent LNCaP cells. Thus, we hypothesize that p27 up-regulation in response to PA may be mediated by alterations in p27 protein stability. To confirm this hypothesis , we performed an *in vitro* ubiquitination assay. p27 ubiquitination, represented by a ladder-type pattern following PA addition, was diminished after 24-hour treatment in all three cell lines (Figure 6). A previously described non-specific band at about 60 kD was seen, probably a crossreacting protein (21). These data may indicate that PA decreases p27-ubiquitin ligation and further degradation by diminishing Skp2 activity in prostate cancer cell lines.

Discussion

Although androgen withdrawal is the most effective therapy for patients with advanced prostate cancer, progression to

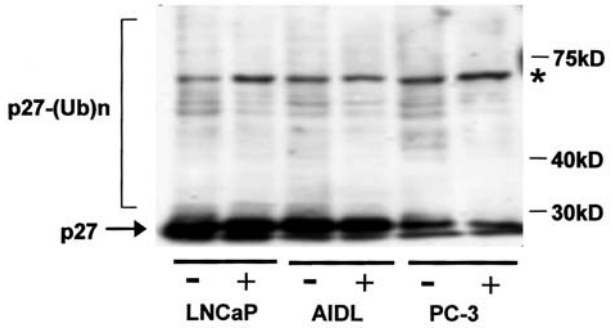

Figure 6. *In vitro ubiquitination of p27. Two mg proteins of whole cell lysates were incubated with ubiquitin affinity beads for 4 hours and the precipitates were subjected to SDS-PAGE, immunoblotted with p27 antibody. Ubiquitinated p27 was decreased in 5 mM PA-treated prostate cancer cells. Asterisk shows non specific bands at about 60 kD.*

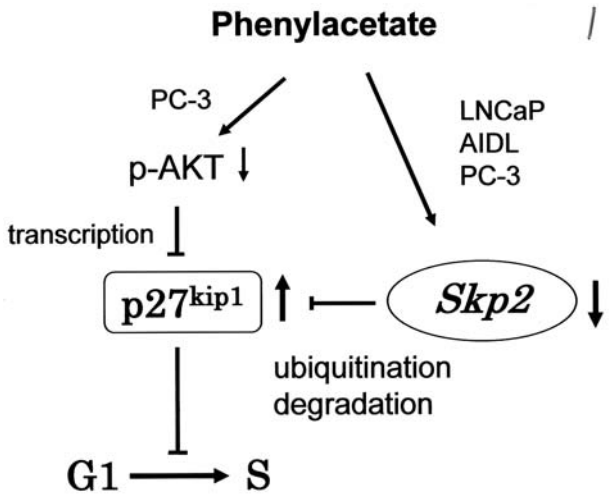

Figure 7. *Proposed model for the pathway of phenylacetate-mediated G1 arrest through p27 in prostate cancer cells. PA decreases Skp2 expression in three prostate cancer cell lines, and phosph-AKT (p-AKT) is down-regulated by PA in only PC-3 cells. Thus, p27 is up-regulated post-translationally in three prostate cell lines, and both transcriptionally and post-translationally in PC-3 cells, followed by G1 arrest.*

androgen independence eventually occurs and remains the main barrier to improved survival and quality of life. Traditional treatment with cytotoxic chemicals is usually ineffective because of low proliferation rates and is limited by toxicity (2). Progression to androgen independence is an intricate process, involving clonal selection and adaptative mechanisms in heterogeneous tumors composed of subpopulations of cells that behave differently to androgen ablation. Thus, it is possible to manipulate these changes triggered by androgen ablation through the adjuvant use of differentiation agents or by modulating the expression of genes involved in the process.

Phenylacetate (PA) has been identified as having antiproliferative effects against several malignant cell types (9-12), however, the molecular mechanism by which PA induces its differentiation and cell cycle arrest has not been elucidated. Our previous report demonstrated the involvement of p27 in PA-mediated cell cycle arrest in prostate cancer cells (13). In the present study, we investigated the mechanism by which PA increased p27 expression in prostate cancer cells. It was reported that Akt activity is associated with transcriptional regulation of p27 in LNCaP (31), however, in this study PA had no effect on p27 mRNA as well as Akt and phospho-Akt, suggesting that PA has little effect on p27 transcription in LNCaP and AIDL cells. Therefore, stabilization in the p27 protein could be responsible for the intracellular accumulation of p27 in PA-treated LNCaP and AIDL cells. Recent reports have strongly suggested that the intracellular concentration of p27 is regulated predominantly by Skp2, an F-box protein, through the ubiquitin-proteasome proteolytic pathway (19, 20). Thus, we hypothesized that the PA-mediated increase in p27 expression could result from the ligand-dependent decrease in Skp2. Our data demonstrated that PA decreased both Skp2 and Cks1 expressions, and p27 ubiquitination. These results suggest that PA decreases p27-ubiquitin ligation and further degradation by diminising Skp2 activity in prostate cancer cells. We believe that PA-induced cell cycle arrest is correlated with inhibition of Skp2-mediated proteolytic degradation of p27 (Figure 7).

The PTEN (phosphatase and tensin homolog deleted in chromosome ten)/MMAC (mutated in multiple advanced cancers) is a tumor suppressor gene associated with multiple tumors, including prostate cancer (32-36). Loss of PTEN/MMAC function can occur through homozygous gene deletion, point mutation, or loss of expression (35). Previous reports have demonstrated that PTEN is mutated in LNCaP and PC-3 cells (37). PTEN negatively regulates the phosphorylation of Akt and increases p27 expression, resulting in the inhibition of the transition from G1- to S-phase of the cell cycle (31, 37). In mouse embryonic stem cells, PTEN increases the p27 protein level and down-regulates the mRNA level of Skp2, but has little effect on transcriptional regulation of p27 and other components of SCF^{skp2} (38). Our results demonstrated that not only p27 mRNA, but also Akt protein and phospho-Akt levels did not change in LNCaP and AIDL, indicating that PA has little effect on transcriptional levels of p27 in these prostate cancer cells. Interestingly, PA increased p27 mRNA levels and decreased Akt phosphorylation in PC-3 cells. These data suggest that PA has effects on the transcriptional

regulation of p27 as well as post-translational regulation in PC-3 cells (Figure 7). Differences in the effect of PA can be related to the androgen-independent status and/or cancer progression. The Akt pathway may play an additive role in the regulation of p27 expression during prostate cancer progression.

Other antiproliferative effects of PA have been reported to be correlated with the peroxisome proliferator-activated receptor γ (PPARγ), which is a member of the nuclear receptor family (39, 40). PPARγ ligands have demonstrated a growth-inhibitory effect on several malignant cell types, including prostate cancer (41-43). PA increases PPARγ expression, interacts directly with the ligand-binding site of PPARγ and activates its transcriptional function in human breast carcinoma and glioblastoma cells (44). PA inhibited cell proliferation and induced differentiation in human neuroblastoma cells through the PPARγ signaling pathway (45). These findings provide evidence for a biological role of PPARγ in tumor cytostasis induced by PA. A decrease in Skp2 expression and a reciprocal increase in p27 expression were found in PPARγ ligand, troglitazone-treated hepatoma cells (43). Thus, it might be one of the candidates of downstream target molecules of PA, although we did not examine whether PA has any effect on PPARγ expressions in prostate cancer cells.

Skp2 is considered to be a protooncogene, and is overexpressed in several human cancers (46-48). Expression of Skp2 alone is sufficient to reduce p27 levels and induce hyperplasia, dysplasia and low-grade carcinomas in the mouse prostate gland in transgenic mouse lines that specifically expressed Skp2 (49). Skp2 is inversely correlated with p27 and a significant correlation was found between Skp2 levels and tumor aggressiveness in prostate cancer (48). Ours and other studies collectively suggest that PA could be a useful therapeutic compound for both androgen-dependent prostate cancer, preventing the hormone-refractory state induced by hormone depletion therapy, and hormone-refractory prostate cancer. In the present study, profound down-regulation of Skp2 and a reciprocal increase of p27 by PA were observed in the more aggressive androgen-independent prostate cancer cell line, PC-3. Thus, a strategy of targeting Skp2 may provide a more selective target for the treatment of advanced prostate cancer.

Acknowledgements

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