Antagonism between PTEN/MMAC1/TEP-1 and Androgen Receptor in Growth and Apoptosis of Prostatic Cancer Cells*

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PTEN/MMAC1/TEP-1 (PTEN) tumor suppressor and androgen receptor play important roles in prostatic tumorigenesis by exerting opposite effects on homeostasis of prostatic epithelium. Here, we describe a mutual repression and selective dominance between PTEN and the androgen receptor (AR) in the growth and the apoptosis of prostatic cancer cells. On the one hand, PTEN and an inhibitor of phosphoinositide 3-kinase repressed the transcriptional activity of the AR as well as androgen-induced cell proliferation and production of prostatic-specific antigen. On the other hand, androgens protected prostate cancer cells from PTEN-induced apoptosis in an AR-dependent manner. Whereas the repression of the transcriptional activity of the AR by PTEN is likely to involve the down-regulation of AKT, androgens protected prostate cancer cells from PTEN-induced apoptosis without an effect on AKT activity, demonstrating a differential involvement of AKT in the interaction between PTEN and the AR. Our data suggest that the loss of PTEN function may induce tumorigenesis through unopposed activity of the AR as well as contribute to the resistance of prostate cancers to androgen ablation therapy.

Androgens are responsible for the development, maintenance, and regulation of male phenotype and reproductive physiology. These activities are mediated through the AR1 (1–4), which belongs to the steroid/thyroid receptor superfamily, a group of ligand-regulated transcription factors (5). Like other members of the family, the AR protein is modular in nature and composed of an amino-terminal A/B region, a DNA-binding domain and a “hinge” region in the middle, and a hormone-binding domain at the carboxyl terminus. Whereas the amino-terminal A/B region contains the major transcriptional activation function, a weaker activation function in the hormone-binding domain also contributes to the total transcriptional activity. The hormone-binding domain represses the activation functions until androgens bind to it and relieve the repression by inducing the formation of a conformation suitable for the interaction with transcriptional cofactors (6–8). In addition, the polymeric stretches within the amino-terminal region (9) and receptor phosphorylation (10, 11) also contribute to the AR transcriptional activities.

Besides their established physiological functions, androgens are implicated in multiple pathological processes including prostate cancer (PCa), which is the most commonly diagnosed malignancy and second only to lung cancer in the mortality rate of American males (12). Chronically maintained androgen level sustains the total prostate cell number by both stimulating the rate of proliferation and inhibiting the rate of death of prostatic epithelial cells (13); both lead to an increase in the cell number. To maintain the homeostasis of prostate epithelium, there must exist a “brake” system that opposes these effects of androgens.

PTEN tumor suppressor is a 403-amino acid phosphoprotein/phospholipid dual-specificity phosphatase (14, 15). Somatic mutation of PTEN is a common event in diverse human cancers including PCa (14, 15), and heterozygous deletion of PTEN in mice leads to neoplasm in multiple tissues including the prostate (16, 17). The significance of the phosphoprotein phosphatase in tumor suppression is largely unknown, although one known substrate is focal adhesion kinase (18). In contrast, multiple findings support a role of the lipid phosphatase activity in PTEN-mediated tumor suppression (19–23). PTEN lipid phosphatase catalyzes the dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) (24), resulting in inactivation of the downstream protein kinase B, also named AKT, activity (21, 22). Ectopic PTEN expression in PTEN-null PCa cells induced cell cycle arrest and apoptosis (22, 25); both activities are opposite to those of androgens, suggesting that PTEN may provide the brake to balance the functions of AR in prostate cells.

In the current study, we provide experimental evidence that the activities of PTEN and AR are antagonistic in PCa cells. Interestingly, PTEN and AR do not simply shut down each others activity but differentially antagonize and selectively dominate each other in PCa cell apoptosis and proliferation.

EXPERIMENTAL PROCEDURES
Plasmids—AREe1bLuc (26), pCMVhAR (27), pLENgal (28), PSA-Luc (29), GalLuc (30), and GalVP16 (6), pSG5L-HA-PTEN-WT (22), pSG5L-HA-PTEN-G129R (22), PLNCX-HA-yr-AKT (22), and PLNCX-HA-yr-AKT179 m (22) have already been described. pCMVβ and pLNCe are from CLONTECH (Palo Alto, CA). pLNEHar was constructed by replacing β-galactosidase (β-gal) cDNA of pLENgal with human AR cDNA. Partial human AR cDNA was excised from pCMVHAR with BamHI and EagI. Because EagI cuts the AR cDNA at an internal position close to the initiator ATG, the digestion yield a partial AR cDNA fragment. The partial AR cDNA was ligated to a synthetic linker that extended the AR cDNA sequence from the internal
Antagonism between PTEN and AR

Fig. 1. PTEN repressed the transcriptional activity of the AR. A, dosage-dependent inhibition of AR activity by PTEN in PC3 cells. Cells were transfected with 0.1 μg of pLENhAR, 0.5 μg of pLENβgal, 0.5 μg of AREe1bLuc, and the indicated amounts of pSG5L-HP-TEN WT (WT) or pSG5L-HP-TEN.G129R (G129R) (MT). Transfected cells were treated for 24 h with 10 μM R1881 or ethanol (EOH) as vehicle controls. All samples were normalized with β-gal activity and expressed as relative luciferase unit (RLU). Duplicate samples were analyzed for each single data point, and the data have been reproduced three times. B, inhibition of AR activity by PTEN was not caused by decreased AR protein levels. PC3 cells were transfected with 0.1 μg of pCMVhAR, 0.5 μg of pCMVB, 0.5 μg of AREe1bLuc, and 0.1 μg of pSG5L-HP-TEN WT or pSG5L-HP-TEN.G129R. The level of AR protein was detected by Western blotting.

with a PTEN expression vector or a control vector expressing a phosphatase-inactive PTEN. The transcriptional activity of AR was measured by assaying the luciferase activity from a co-transfected AR reporter, AREe1bLuc, in which luciferase expression is under the control of synthetic androgen response elements (AREs) placed in front of the small adenovirus E1b promoter. The effect of PTEN on the AR activity was measured by comparing the AR activity in cells transfected with PTEN to those transfected with the control vector. Because of the concern that co-transfected PTEN may alter the expression of AR by affecting the activity of the promoter or the enhancer of the AR expression vector, the reporter activity was normalized with β-gal activity from co-transfected pLENβgal vector in which β-gal expression is under the control of human metallothionin-II promoter and SV40 enhancer, the same regulatory sequences used by the AR vector. As shown in Fig. 1A, AR basal activity was low but the activity was significantly induced by treatment with R1881, a stable synthetic androgen agonist. Whereas the basal activity was not affected, R1881-induced AR activity was repressed, but not abolished, by PTEN in a dosage-dependent manner.

Because both AR and β-gal were expressed using the same vector, the way we normalize the reporter activity should have eliminated the potential interference caused by either variation in transfection efficiency or PTEN-induced alterations in the promoter activity of the AR expression vector. However, the lower AR activity in the presence of PTEN could argue either because of lower level of AR protein as a result of decreased protein stability or reduced efficiency of protein translation. To test whether the level of AR protein was altered by PTEN, PC3 cells were transfected with pCMVBhAR and pCMVBβ, which express the receptor and β-gal, respectively, under the control of the stronger cytomegalovirus (CMV) promoter, permitting the

Repression of AR Transcriptional Activity by PTEN and a PI3K Inhibitor and Relief of the Repression by a Dominantly Active AKT—To test whether PTEN inhibits AR transcriptional activity, we transfected into AR-negative, PTEN-null PC3 cells (22) an AR expression vector, pLENhAR, together
Antagonism between PTEN and AR

Fig. 2. PTEN repressed the androgen-induced increase in AR activity over time. PC3 cells were transfected with 0.1 μg of pCMVhAR, 0.5 μg of pCMVβ, 0.5 μg of AREe1bLuc, and 0.1 μg of pSG5L-HA-PTEN-WT or pSG5L-HA-PTEN(G129R). Transfected cells were treated for indicated times with 10^{-8} M R1881 or ethanol (EOH) as vehicle controls. AR activity was assayed and expressed as described in the legend to Fig. 1.

So far, we have demonstrated an inhibitory effect of PTEN and the PI3K inhibitor on androgen-induced PSA production and cell proliferation with transiently transfected AR with a reporter constructed with synthetic AREs. To determine whether PTEN also represses endogenous AR activity with natural promoters, we transfected PSALuc into PTEN-null but AR-positive LNCaP cells (22) and examined the effect of PTEN and the PI3K inhibitor on endogenous AR activity. PSALuc is an AR reporter in which luciferase expression is under the control of the promoter of human PSA, a PCa marker, which is transcriptionally regulated by AR through complex AREs (29). As shown in Fig. 5A, the transcriptional activity of the endogenous AR in cells transfected with the PSALuc was increased by R1881, and R1881 induction was blocked by PTEN. This demonstrates that PTEN represses endogenous AR activity on natural promoters and thus is not limited to ectopic AR or synthetic AREs.

To determine the biological consequence of the observed repression of AR transcriptional activity, the effect of PI3K inhibitor on androgen-induced PSA production and cell proliferation was examined in LNCaP cells. LNCaP cells were treated with either R1881 alone or co-treated with LY294002 for indicated times. Culture medium from the treated cells was collected and analyzed for PSA level by ELISA. As shown in Fig. 6A, R1881 treatment stimulated PSA production and the stimulation was inhibited by the co-treatment with LY294002. Sim-
Antagonism between PTEN and AR

Fig. 4. A dominantly active AKT blocked PTEN-induced AR repression. PC3 cells were transfected with 0.1 μg of pLENHar, 0.5 μg of AREe1bLuc, 0.5 μg of pLENGal, 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R and 0.1 μg of PLNCX-HA-myr-Akt (Active) or PLNCX-HA-myr-AKT179M (Inactive). AR activity was assayed and expressed as described in the legend to Fig. 1.

Fig. 5. PTEN repressed the transcriptional activity of endogenous AR in LNCaP cells. A. PTEN repression of endogenous AR activity on PSA promoter in LNCaP cells. Cells were transfected with 0.5 μg of PSELuc, 0.5 μg of pLENGal, and 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R. Transfected cells were treated with or without 10^-8 M R1881, and AR activity was assayed and expressed as described in the legend to Fig. 1. B. Lack of Gal-VP16 repression by PTEN in LNCaP cells. Cells were transfected with 0.5 μg of GalLuc, 0.5 μg of pCMVβ, 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R and with or without 0.1 μg of Gal-VP16. Transfected cells were treated with 10^-8 M R1881. Transcriptional activity of VP16 was assayed and expressed as described in the legend to Fig. 1.

Similar to PSA production, R1881 induced an increase in cell number as measured by MTT assays, and this increase was blocked by co-treatment with LY294002 (Fig. 6B). LY294002 did not consistently decrease the basal PSA level or LNCaP cell numbers in the absence of R1881. These analyses demonstrate that the repression of AR transcriptional activity by the PI3K inhibitor impaired the biological functions of endogenous AR.

AR-dependent Protection of PTEN-induced Apoptosis by Androgens in PCa Cells—Our data so far have established a functional relationship between PTEN and AR by showing that PTEN or a PI3K inhibitor opposed AR function in PCa cells. We next investigated whether androgen also opposes the function of PTEN. Because the expression of PTEN in LNCaP cells induced apoptosis (25), we examined the effect of androgens on PTEN-induced apoptosis. LNCaP cells were transfected with a green fluorescence protein (GFP) expression vector and the PTEN expression vector or the control vector expressing the mutant PTEN. The transfected cells were then treated with or without R1881, and the viability of transfected cells was determined. As shown in Fig. 7A, the viability of PTEN-transfected cells in the absence of R1881 was only about 15% of the cells transfected with the control vector. Treatment with R1881 restored the viability of PTEN-transfected cells to the level of controls. This decrease in the viability of PTEN-transfected cells as well as its blockage by R1881 was more directly shown as its blockage by R1881 was more directly shown.

Fig. 6. PI3K inhibitor impaired the biological activities of the AR. A. Repression of androgen-induced PSA production in LNCaP cells by LY294002. LNCaP cells were starved and treated with vehicle, 10^-8 M R1881 or 10^-8 M R1881 plus 20 μM LY294002 for the indicated times. Absolute amounts of Me2SO and ethanol were the same for all samples. PSA levels were quantified by ELISA. Duplicate samples were analyzed for each data points, and the experiment was repeated three times. B. Repression of androgen-induced LNCaP cell proliferation by LY294002. LNCaP cells were starved and treated as in A. Cell numbers were determined by the MIT colorimetric assays. Eight samples were analyzed for each data point, and the data were reproduced three times.

Fig. 7. Androgen protected LNCaP cells from PTEN-induced apoptosis. A. Androgen effect on the viability of PTEN-transfected LNCaP cells. LNCaP cells were transfected with 0.5 μg of pLNCe, a GFP expression vector and 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R. Transfection cells were treated with 10^-8 M R1881 or ethanol for 24 h, and the viability of transfected cells in each well was determined by counting the total number of green cells. Triplicate samples were analyzed for each data point, and the data were reproduced three times. B, Representative micrographs of PTEN-transfected cells in the presence or absence of androgen. Cells were transfected and fixed as in A. Fixed cells were stained with DAPI, and representative micrographs were captured by a CCD camera attached to the fluorescence microscope using objective lens of ×10 (panels 1–3) or ×100 (panels 4–6). C, Androgen effect on PTEN-induced increase in apoptotic index of LNCaP cells. Cells were processed as in B, and the apoptotic index of GFP-positive cells was determined by scoring 300 GFP-positive cells for chromatin condensation. Triplicate samples were analyzed per data point, and the graph represents three independent experiments.
Antagonism between PTEN and AR

Fig. 8. Androgen effect on PTEN-induced apoptosis was AR-dependent. A, AR-dependent androgen effect on PTEN-induced decrease in PC3 cell viability. PC3 cells were transfected with 0.5 μg of pLNEC, 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R and with or without 0.1 μg of pLENhAR. Transfected cells were treated and processed for cell viability determination as described in the legend to Fig. 7. B, effect of androgen on PTEN-induced increase in apoptotic index in AR-transfected PC3 cells. Cells were transfected with AR and PTEN expression vectors and processed as in A. Apoptotic index was scored as described in the legend to Fig. 7. C, androgen protection of PTEN-induced PC3 cell death over time. Cells were transfected with AR and PTEN expression vectors as in A and treated with 10−8 M R1881 or ethanol for indicated times. The viability of transfected cells in each well was determined as described in the legend to Fig. 7.

Fig. 9. Androgen protection of PTEN-induced apoptosis in LNCaP cells occurred independent of AKT activity. A, inhibition of PTEN-induced apoptosis by dominantly active AKT. PC3 cells were transfected with 0.5 μg of pLNEC, 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R and 0.5 μg of PLNCX-HA-myr-AKT or PLNCX-HA-myr-AKTKI79M. The viability of transfected cells was determined as described in the legend to Fig. 7. B, lack of androgen effect on PTEN-induced down-regulation of AKT activity. LNCaP cells were transfected with 0.5 μg of PLNCX-HA-AKT (wtAKT) and 0.1 μg of pSG5L-HA-PTEN:WT or pSG5L-HA-PTEN:G129R. Transfected cells were treated with 10−8 M R1881 or vehicle for 24 h. Both AKT kinase activity (top panel) and level of expression (bottom panel) were analyzed by parallel immunocomplex kinase assays and Western blotting using the 12CA5 anti-HA monoclonal antibody. Phosphorylated histone H2b (P-H2b) was resolved on a 15% SDS-PAGE gel and visualized by autoradiography.

by the number of GFP-positive cells in representative micrographic fields (Fig. 7B, panels 1–3).

To confirm that the decreased viability of PTEN-transfected cells is the result of cell apoptosis, cells were fixed after transfection and stained with DAPI, and the nuclear morphology of transfected cells was examined for features of apoptosis under a fluorescence microscope that permits the simultaneous visualization of both blue and green fluorescence. As shown in Fig. 7B, panels 4–6, as representative micrographs, cells transfected with the control vector displayed a normal morphology similar to surrounding non-transfected cells (Fig. 7B, panel 4). Cells transfected with PTEN without R1881 treatment frequently displayed an apoptotic morphology (Fig. 7B, panel 5). Similar to controls, most cells transfected with PTEN but treated with R1881 showed a normal morphology (Fig. 7B, panel 6). Apoptotic index, as determined by counting apoptotic cells in 300 green cells per sample 24 h after treatment, was 5% for controls, 20% for cells transfected with PTEN without R1881 treatment, and 5% for cells transfected with PTEN but treated with R1881 (Fig. 7C). These analyses show that PTEN-induced apoptosis in LNCaP cells, and this PTEN function was blocked by androgen treatment.

Because of the presence of endogenous AR in LNCaP cells, LNCaP cell experiments did not show that the androgen protection of PTEN-induced apoptosis is mediated through the AR. To determine whether the androgen effect on apoptosis is AR-dependent, AR-negative PC3 cells were transfected with or without AR, and the effect of androgen on PTEN-induced apoptosis was examined in LNCaP cells. As shown in Fig. 8A, R1881 had no effect on the viability of PTEN-transfected cells in the absence of ectopic AR expression. After co-transfection with AR, both PTEN-induced decreases in cell viability (Fig. 8A) and increases in apoptotic index (Fig. 8B) were blocked by R1881, demonstrating that the androgen protection of PTEN-induced apoptosis was mediated through the AR.

To further analyze the anti-apoptotic function of AR, a time course study was performed (Fig. 8C). In this study, the number of transfected (green) cells peaked at 24 h post-transfection, and R1881 protected PC3 cells from PTEN-induced death similarly at all three tested time points, presumably because of the synchronized expression of AR and PTEN proteins in co-transfected cells.

Lack of an Androgen Effect on AKT Activities in PCa Cells—Our data in Fig. 1 showed that the PTEN repression of AR depended on the down-regulation of AKT activity. In addition, co-expression of the dominantly active AKT blocked PTEN-induced apoptosis in LNCaP cells (Fig. 9A), demonstrating that PTEN-induced apoptosis was mediated through AKT down-regulation. So we investigated the possibility that androgens might protect apoptosis by regulating AKT activity. Western blotting with anti-[phospho-Ser473]AKT antibody did not detect an androgen effect on endogenous AKT activity in LNCaP cells (data not shown).

LNCaP cells are PTEN-null and contain high levels of endogenous AKT activity. Although androgens do not have a direct effect on endogenous AKT activity, it may affect the negative regulation of AKT by PTEN. So we next examined whether androgen treatment blocked PTEN-induced AKT...
down-regulation. We transfected a HA-tagged wild-type AKT into LNCaP cells with the PTEN expression vector and analyzed the AKT activity by in vitro immunocomplex kinase assays. As shown in Fig. 9B, in either the presence or absence of R1881, the kinase activity of AKT was dramatically decreased by PTEN expression whereas the level of AKT protein was slightly reduced (Fig. 9B). The data demonstrate that R1881 did not block the down-regulation of AKT activity by PTEN in LNCaP cells.

**Discussion**

Our studies demonstrated an antagonistic interaction and selective dominance between PTEN and AR in the proliferation and apoptosis of PCa cells as well as a differential involvement of AKT in the interaction. The lack of an androgen effect on both AKT activity and its down-regulation by PTEN in PCa cells suggests that androgens protect PTEN-induced apoptosis either by activating a survival pathway that is independent of AKT as suggested by a previous study (33) or by activating the same survival pathway at steps downstream of AKT.

It appears paradoxical that PTEN-or LY294002-induced repression of AR transcriptional activity was sufficient to block androgen-induced proliferation and PSA production but unable to override the protective effect of androgens on apoptosis. One possibility is that AR-dependent protection of apoptosis by androgens might be mediated through non-genomic effects of the receptor. Our data clearly showed that the androgen effect on PTEN-induced apoptosis is AR-dependent. Consistent with our data, reported studies demonstrated that the "decoy" of ARE triggered apoptosis in LNCaP cells (34), suggesting that the anti-antagonist effect of androgens is mediated through the genomic effect of the AR. Because the AR transcriptional activity was repressed but not abolished by PTEN or LY294002 in our experiments, it is likely that the androgen induction of genes involved in promoting cell proliferation, and those in apoptosis protection require different amounts of AR transcriptional activity.

Because our studies indicate that androgen target genes involved in proliferation and apoptosis protection may have a differential sensitivity to cellular status of PI(3,4,5)P3 signal pathway, it would be interesting to determine whether PTEN signaling in prostate epithelial cells to unopposed AR activity. Similarly, excessive androgens may induce prostatic tumorigenesis by blocking the apoptosis-promoting function of PTEN. The induction of apoptosis by the restored PTEN expression in LNCaP cells only occurred in the absence of androgen, implying that PTEN mutation or decreased expression may contribute to the resistance of PCa to androgen ablation and that the combinational inhibition of both PI3K/AKT and androgen signals could be an effective approach for the treatment of AR-positive PCa.

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Antagonism between PTEN and AR

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