Increased AKT Activity Contributes to Prostate Cancer Progression by Dramatically Accelerating Prostate Tumor Growth and Diminishing p27Kip1 Expression*

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The PTEN tumor suppressor gene is frequently inactivated in human prostate cancers, particularly in more advanced cancers, suggesting that the AKT/protein kinase B (PKB) kinase, which is negatively regulated by PTEN, may be involved in human prostate cancer progression. We now show that AKT activation and activity are markedly increased in androgen-independent, prostate-specific antigen-positive prostate cancer cells (LNAI cells) established from xenograft tumors of the androgen-dependent LNCaP cell line. These LNAI cells show increased expression of integrin-linked kinase, which is putatively responsible for AKT activation/Ser-473 phosphorylation, as well as for increased phosphorylation of the AKT target protein, BAD. Furthermore, expression of the p27Kip1 cell cycle regulator was diminished in LNAI cells, consistent with the notion that AKT directly inhibits AFX/Forkhead-mediated transcription of p27Kip1. To assess directly the impact of increased AKT activity on prostate cancer progression, an activated hAKT1 mutant was overexpressed in LNCaP cells, resulting in a 6-fold increase in xenograft tumor growth. Like LNAI cells, these transfectants showed dramatically reduced p27Kip1 expression. Together, these data implicate increased AKT activity in prostate tumor progression and androgen independence and suggest that diminished p27Kip1 expression, which has been repeatedly associated with prostate cancer progression, may be a consequence of increased AKT activity.

The molecular alterations that facilitate human prostate cancer (hPCA) progression and the emergence of androgen-independent tumor cells are unclear but may involve a progressive decrease in the normal apoptotic response (1–3) as well as a release from the cell cycle arrest that follows androgen withdrawal (4). Indeed, hPCA progression has been associated repeatedly with decreased expression of the cell cycle regulator p27Kip1 (4–9).

The PTEN/MMAC tumor suppressor gene is frequently inactivated in primary human prostate cancers, particularly in the more advanced cancers (10), and in human prostate cancer xenografts and cell lines including PC-3, Du145, and LNCaP (11–13). These studies suggest that components of the phosphatidylinositol 3-kinase pathway that are negatively regulated by PTEN, such as the key cell survival kinase AKT (14–16), may be increasingly activated with prostate tumor progression. Indeed, activated AKT regulates a number of intracellular targets implicated in prostate tumor progression and androgen independence. For instance, AKT-dependent inactivation of pro-apoptotic proteins such as BAD and caspase-9 (17, 18) may suppress the normal apoptotic response. Additionally, AKT may enhance cell cycle progression by suppressing AFX/Forkhead transcription factor activity (19–22), which would result in diminished expression of AFX target genes such as the cell cycle inhibitor p27Kip1 (23). Furthermore, AKT can elicit enhanced translation of key growth-regulatory proteins, like cyclin D1 (24), by stimulating FRAP/mTOR kinase activity and de-repressing translation initiation (Refs. 25 and 26; depicted in Fig. 1).

Because AKT is a central regulator of many intracellular processes implicated in prostate tumor progression and because PTEN, the negative regulator of AKT, is functionally inactivated in a significant proportion of advanced hPCAs, we explored whether increased AKT activity may be involved in prostate tumor progression and androgen independence. To better model androgen-independent hPCAs, we established a novel series of AR⁺, PSA⁺, androgen-independent hPCAs (LNAI cells) derived from xenograft tumors of the androgen-sensitive/deep LNCaP prostate cancer cell line. Our analyses of these cells reveals that AKT activity is increased with androgen-independent progression corresponding to increased expression of integrin-linked kinase, the kinase putatively responsible for the activation of AKT by phosphorylation at Ser-473 (Refs. 27 and 28; Fig. 1), increased phosphorylation of the pro-apoptotic protein BAD and markedly reduced expression of the cell cycle regulator p27Kip1. In addition, overexpression of an activated AKT-1 cDNA in LNCaP cells dramatically accelerates xenograft tumor growth and suppresses p27Kip1 expression. These data implicate increased AKT activity in human prostate cancer progression and androgen independence and
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EXPERIMENTAL PROCEDURES

Cell Culture and Establishment of Cell Lines ex Vivo—Cells were cultured in phenol red-free RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). All xenograft studies were performed by injecting 4 × 10^6 cells subcutaneously in 100 μl of a 1:1 mix of 1× phosphate-buffered saline and Matrigel (Becton Dickinson, Bedford, MA) into castrated male nude mice (Harlan Sprague-Dawley). First-generation LNAI cell lines, LNAI.T1 and LNAI.T2, were derived from a xenograft tumor of LNCaP established in an intact mouse that continued to grow following castration. Second generation LNAI cells, designated T1.8, T1.16, T2.9, T2.10, and T2.11, formed tumors more rapidly in both intact and castrated mice than the first-generation LNAI cells, LNAI.T1 and LNAI.T2, respectively, growing in castrated mice. To evaluate tumor formation and growth in the presence or absence of circulating androgen, LNAI.T2, were derived from a xenograft tumor of LNCaP established in intact mice (Harlan Sprague-Dawley). Prostate Cancer Cell Lines—The majority of androgen-independent hPCa express both the androgen receptor and prostate-specific antigen (PSA). The most widely available and commonly used androgen-independent human prostate cancer cell lines, PC-3 and DU145, lack expression of both AR and PSA and therefore may not reflect the majority of advanced, androgen-independent hPCa. To better model androgen-independent prostate cancer, we developed a panel of cell lines from the AR^+, PSA^+ androgen-independent human prostate cancer cell lines, PC-3 and DU145, carrying the cDNA for the activated mutant of human AKT-1^T308D/S473D. LNCaP cells were selected in culture media supplemented with 190 units/ml hygromycin (CalBiochem, San Diego, CA).

RESULTS

Establishment of AR^+, PSA^+ Androgen-independent Human Prostate Cancer Cell Lines—The majority of androgen-independent hPCa express both the androgen receptor and prostate-specific antigen (PSA). The most widely available and commonly used androgen-independent human prostate cancer cell lines, PC-3 and DU145, lack expression of both AR and PSA and therefore may not reflect the majority of advanced, androgen-independent hPCa. To better model androgen-independent prostate cancer, we developed a panel of cell lines from the AR^+, PSA^+, androgen-dependent/hPCa cell line, LNCaP, following in vivo selection for xenograft tumor growth persisting after castration. These cell lines, designated LNAI cells, readily formed tumors both in intact, testosterone-supplemented male nude mice and in castrated male nude mice (Table I), indicating that these cell lines are androgen-independent. Notably, the second-generation LNAI cells, T1.8, T1.16, T2.11, formed tumors more rapidly in both intact and castrated hosts than the first-generation LNAI cells, LNAI.T1 and LNAI.T2. All of these LNAI cells expressed PSA (Fig. 2) and AR (data not shown). PSA was also evident in the serum from LNAI xenograft-bearing intact and castrated mice (Fig. 2) and AR (data not shown). Therefore, we have successfully established androgen-independent hPCa cells that, like the majority of androgen-independent hPCa, retain expression of both AR and PSA and share the same genetic background as the androgen-depend-
Tumor cells (4 x 10^5) were injected subcutaneously into the left flank of intact, testosterone-supplemented or castrated male nude mice in a 1:1 mix or 1 x phosphate-buffered saline and matrigel in 100 μl total volume. Tumor formation was monitored weekly. Latency period represents the time (days post-injection) to a definable tumor (>100 mm^3) ± S.E. Time (days post-injection) to 1300 mm^3 is indicated ± S.E.

| Cell line     | Tumor incidence | Latency period | Time to 1300 mm^3 |
|---------------|-----------------|----------------|------------------|
|               | Intact | Castrate | Intact | Castrate | Intact | Castrate |
| LNCaP         | 7/7    | 0/5     | 51 ± 6 | N/A      | N/A    | N/A     |
| LNAI.T1       | 5/5    | 4/5     | 43 ± 0 | 45 ± 2   | 53 ± 3 | 86 ± 6  |
| LNAI.T1.8     | 5/5    | 4/5     | 34 ± 4 | 31 ± 4   | 53 ± 9 | 69 ± 9  |
| LNAI.T1.16    | 5/5    | 4/5     | 29 ± 4 | 31 ± 4   | 49 ± 5 | 45 ± 6  |
| LNAI.T2       | 4/5    | 2/5     | 51 ± 5 | 97 ± 0   | N/A    | N/A     |
| LNAI.T2.10    | 5/5    | 5/5     | 29 ± 4 | 40 ± 5   | 53 ± 5 | 88 ± 8  |
| LNAI.T2.11    | 4/5    | 5/5     | 26 ± 2 | 32 ± 6   | 62 ± 9 | 77 ± 8  |

* These data were pooled from two experiments. LNCaP tumors did not grow to 1300 mm^3 within 100 days-post injection. N/A, not applicable.

FIG. 2. LNAI cells express prostate-specific antigen. Expression of PSA was evaluated in LNCaP or LNAI cell lysates by Western blot analyses (20 μg/lane). Recombinant human PSA (rPSA) was included as a positive control for PSA immunoblotting. These data are representative of three independent experiments.

FIG. 3. AKT activation and activity are increased in LNAI cells. A, activation of AKT was monitored by immunoblotting for AKT^Ser473 phosphorylation in LNCaP and LNAI cell lysates (20 μg/lane). Immunoblotting for total AKT protein was performed simultaneously on a duplicate blot. To control for gel loading and transfer, blots were routinely reprobed for β-actin. These immunoblots are representative of six independent experiments. B, AKT activity was assessed as described under “Experimental Procedures” following immunoprecipitation of AKT from 200 μg of cell lysate. Data are shown for LNCaP, ALT1, T1.16, and cells derived from a xenograft tumor of T1.16 (T1.16X). These data are representative of four independent experiments.

FIG. 4. RT-PCR analyses for expression of AKT-1, AKT-2, and AKT-3. RT-PCR analyses using primers specific for each AKT isoform (33) were run for 35 PCR cycles. The product sizes, in base pairs, are 330 for AKT-1, 335 for AKT-2, and 327 for AKT-3. AKT-3 RT-PCR products was verified by restriction digestion with HaeIII and NotI (data not shown). C, expression of AKT-1 and AKT-3 in tissue from normal prostate, prostate cancer, and prostate cancer metastases to bone and soft tissues. Note that each of these samples was positive for β2-microglobulin by RT-PCR (data not shown).
**A) Cell Lines**

| ILK     | LNCaP | ALT1 | T1.8 | T1.16 | ALT2 | T2.11 |
|---------|-------|------|------|-------|------|-------|
| β-actin |       |      |      |       |      |       |

**B) Xenograft Tissues**

| LNCaP | LNAI Xenografts |
|-------|----------------|
| β-actin |           |

**Fig. 5. Integrin-linked kinase expression is increased in LNAI cells.** Expression of ILK was assessed by Western blotting of cell culture extracts (A) or extracts from individual xenografts (B) (20 μg/ lane). Blots were reprobed for expression of β-actin to control for loading and transfer. Specific bands for the 59-kDa ILK and the 46-kDa β-actin proteins are indicated. These blots are representative of three independent experiments each with the cell culture and xenograft lysates. Note that each of the LNAI xenograft tissues has high level expression of ILK, whereas only one of the four LNCaP xenograft tissues shows high level ILK expression.

**Fig. 6. BADSer-136 phosphorylation is increased in LNAI cells and xenograft tissue.** Phosphorylation of BAD was detected by Western blotting with a BADSer-136 phospho-specific antibody (30 μg extract/ lane). Parallel Western blots were also run and immunoblotted for total BAD protein. Signal intensity for pBADSer-136 was normalized for BAD expression. The data presented are from a single experiment and are representative of six independent experiments with the cell culture lysates (upper panel) and three experiments with xenograft tissue lysates (lower panel). In the lower panel, each bar represents an individual xenograft tumor. In both panels, hatched bars represent LNAI lysates, and the clear bars represent LNCaP lysates.

**Fig. 7. p27Kip1 expression is diminished in LNAI cells.** Expression of the cell cycle regulatory protein p27Kip1 was examined by Western blotting (30 μg lysate/lane). Data presented are representative of four independent experiments.

Expression of ILK was increased in androgen-independent LNAI cells compared with androgen-dependent/sensitive LNCaP cells. The pro-apoptotic protein BAD can be functionally inactivated by phosphorylation at Ser-136 by AKT (17, 36, 37). Xenograft tissue and cell culture extracts of LNAI showed increased phosphorylation of BAD at the AKT target site, Ser-136, relative to LNCaP cell and xenograft extracts (Fig. 6).

AKT negatively regulates AFX-mediated transcription of the cell cycle regulator p27Kip1 (23), inhibition of AFX-mediated transcription by AKT may lead to decreased expression of p27Kip1. In LNAI cells, in which AKT is hyperactivated, the protein expression of p27Kip1 was markedly reduced relative to that in LNCaP cells (Fig. 7). This decrease was most pronounced in the most aggressively growing, second-generation LNAI cells with the highest AKT activity (e.g. T1.16). These data are consistent with the notion that AKT negatively regulates AFX-mediated transcription of p27Kip1 but do not exclude the possibility that other mechanisms, such as decreased protein or RNA stability, may contribute to p27Kip1 loss. Together, these data indicate that the increased AKT activity in androgen-independent LNAI cells profoundly effects key downstream mediators involved in cell cycle arrest and apoptotic signaling.

**Stable Overexpression of Constitutively Active AKT Dramatically Enhances LNCaP Xenograft Tumor Growth in Intact Male Nude Mice—**Our data indicate that AKT activity is markedly enhanced in androgen-independent LNAI cells compared with androgen-dependent/sensitive LNCaP and suggest that increased AKT activity may be intimately involved in the progression of hPCa and the emergence of androgen-independent hPCa cells. To determine directly how AKT may impact upon hPCa progression, we established stable overexpressors of the constitutively active AKT<sup>Ser-473</sup> mutant in LNCaP cells (LNCaP:AKT<sup>DD</sup> cells) (Fig. 8, inset). Subcutaneous injection of these LNCaP:AKT<sup>DD</sup> cells into intact, testosterone-supplemented male nude mice yielded rapidly growing tumors with 100% incidence (4/4) in less than 30 days post-injection, whereas vector control cells formed tumors in only three of five mice that were not evident until day 37 post-injection (Fig. 8). Moreover, the LNCaP:AKT<sup>DD</sup> cells showed a dramatic increase in tumor growth (Fig. 8), with a mean tumor volume more than 6-fold that of the vector control transfectants at all time points (by Dunnett’s test on ranked tumor volumes, p < 0.05 for all time points). Like the aggressively growing LNAI cells, LNCaP:AKT<sup>DD</sup> tumor cells showed a substantial decrease in p27Kip1 expression (Fig. 8, inset). Tumors did not form in castrated mice following injection with either LNCaP:AKT<sup>DD</sup> or vector control LNCaP cells. A second, independent experiment confirmed that LNCaP:AKT<sup>DD</sup> cells formed more rapidly growing tumors in intact male nude mice when compared with vector control LNCaP cells but were unable to form tumors in castrated male nude mice (data not shown). Therefore, although insufficient to elicit growth in castrated male nude mice, overexpression of the constitutively active AKT<sup>DD</sup> dramatically enhanced LNCaP xenograft tumor growth in intact male nude mice and resulted in markedly reduced expression of p27Kip1.

**DISCUSSION**

We have developed a series of androgen-independent hPCa cell lines (LNAI cells) from the androgen-dependent/sensitive hPCA cell line, LNCaP. Like the majority of androgen-independent hPCa cells (34), these LNAI cells retain expression of AR and PSA. We have now shown that AKT activation (Ser-473 phos-
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Fig. 8. Xenograft growth of LNCaP:AKT transfectants in intact male nude mice. LNCaP:AKTDD or LNCaP vector control cells were injected (4 \times 10^6 cells) into intact, testosterone-supplemented male nude mice. Tumor volume (mm^3) is depicted on the y-axis and was calculated as described (see "Experimental Procedures"). Hatched bars represent the tumor volumes for individual LNCaP:AKTDD tumors, whereas white bars represent individual LNCaP vector control tumors. LNCaP:AKTDD formed tumors in four of four mice injected, whereas the LNCaP vector control formed tumors in only three of five mice injected. The inset shows Western blot analyses (20 \mu g extract/lane) in LNCaP:AKTDD transfectants and vector control LNCaP transfectants for expression of AKT (representative of three independent experiments) and p27Kip1 (representative of six independent experiments). To control for gel loading and transfer, these blots were reprobed for \beta-actin expression. A second xenograft experiment showed a similar increase in LNCaP:AKTDD xenograft growth relative to the LNCaP vector control (data not shown).

phorylation) and activity are increased in these LNAI cells when compared with androgen-dependent LNCaP cells and may be a consequence of increased expression of the integrin-linked kinase, the kinase putatively responsible for AKT3 phosphorylation (27, 28). Like parental LNCaP cells, LNAI cells express only AKT-1 and AKT-2, indicating that AKT-3 expression is not necessary for androgen independence, as has been suggested in studies with the androgen-independent PC-3 and Du145 hPCa cells (32). Moreover, our data reveal that AKT-3 is expressed not only in prostate cancer tissues but also in normal prostate tissue, indicating that AKT-3 expression is not induced in, or restricted to, prostate malignancy. We have also shown that the increased AKT activity in these LNAI cells corresponds to increased phosphorylation of the pro-apoptotic protein, BAD, and markedly decreased expression of the p27Kip1 cell cycle regulator, consistent with the notion that AKT inhibits AFX-mediated transcriptional activation of p27Kip1 (23, 38). Taken together, these data imply increased AKT activity in hPCa progression and the emergence of androgen-independent human prostate cancer cells.

To address whether AKT alone can drive prostate tumor progression and/or the emergence of androgen-independent cells, we established LNCaP cells with stable overexpression of a constitutively active AKT-1 cDNA. Though alone insufficient to elicit xenograft tumor growth in castrated mice, overexpression of activated AKT dramatically increased tumor growth in intact mice relative to vector control transfectants. Like the most aggressively growing LNAI cells (e.g. T1.16), these LNCaP:AKTDD cells also showed a remarkable reduction in p27Kip1 expression, suggesting that p27Kip1 loss, which has been repeatedly linked to hPCa progression (4–9), may be a consequence of increased AKT activity. Taken together with the analyses of LNAI cells, these data reveal that increased AKT activity contributes to hPCa progression by dramatically accelerating prostate tumor growth, perhaps in relation to decreasing p27Kip1 expression, and is specifically associated with the more advanced, aggressively growing androgen-independent hPCa cells (e.g. T1.16).

Increased AKT activity may play a profound role in the progression of human prostate cancers. AKT regulates many of the processes associated with metastatic progression and the emergence of androgen-independent hPCa cells, such as a diminished apoptotic response (1, 2, 39) as well as a release from the cell cycle control that follows androgen ablation (4). AKT can dampen the normal apoptotic response by suppressing the activity of numerous pro-apoptotic proteins including BAD, caspase 9, and the Forkhead family of transcription factors (Refs. 17–22; summarized in Fig. 1). AKT can also facilitate the release of cells from cell cycle control by inhibiting AFX/Forkhead-mediated translation of the key cell cycle regulator, p27Kip1 (23). Further, by activating FRA1/mTOR and de-repressing translation initiation, AKT may specifically enhance the translation of cyclin D1 (24) as well as other translationally controlled growth factors and growth regulatory proteins implicated in human prostate cancer progression (Refs. 40–43; see Fig. 1).

The data in this report clearly imply increased AKT activity in hPCa progression. The most aggressively growing, androgen-independent LNAI cells had the greatest increase in AKT activity. Moreover, overexpression of an activated AKT-1 mutant dramatically accelerated LNCaP xenograft tumor growth. However, our data also reveal that overexpression of an activated AKT-1 was alone insufficient to drive xenograft tumor growth in castrated mice, suggesting that factors in addition to increased AKT activity must contribute to the ability of LNAI cells to form and grow tumors in the absence of circulating androgens. For instance, the increased expression of ILK in LNAI cells may influence alternate pathways in addition to the AKT pathway, such as the Wnt pathway (27), that may contribute to the androgen-independent phenotype. Moreover, other intracellular pathways may be activated in LNAI cells independent of, or upstream from, AKT that contribute to the androgen-independent phenotype of LNAI cells. Indeed, activation of the mitogen-activated protein kinase pathways, perhaps as a downstream consequence of HER2 activation, has been implicated in hPCa progression and androgen independence (44–46).

In summary, the data presented in this report indicate that increased AKT activity, perhaps as a consequence of increased ILK expression, is involved in hPCa progression and suggest that the reduction in p27Kip1 expression, which has been repeatedly associated with hPCa progression and androgen independence (4–9), may be a consequence of enhanced AKT activity. Together with previously published reports that PTEN is inactivated in a significant proportion of advanced primary human prostate cancers (10) and that AKT regulates many of the intracellular processes associated with hPCa progression (1, 25), the data in this report implicate AKT as a key mediator of prostate tumor progression and suggest that AKT may be a prime target for prostate cancer therapy.

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