The influence of growth hormone/insulin-like growth factor deficiency on prostatic dysplasia in pbARR2-Cre, PTEN knockout mice

K Takahara1,2,6, N Ibuki1,6, M Ghaffari1,3, H Tearle1, CJ Ong1, H Azuma2, ME Gleave1,4, M Pollak5 and ME Cox1,4

BACKGROUND: Elevated insulin-like growth factor-I (IGF-I) serum levels and phosphatase and tensin homolog (PTEN) loss are prostate cancer (PCa) risk factors that enhance androgen-responsive and castration-resistant PCa xenografts growth.

METHODS: The impact of suppressed growth hormone (GH)/IGF-I levels on neoplastic initiation of PTEN-deficient prostate epithelia was assessed histologically and by epithelial-to-mesenchymal marker expression in Ghrhr D60G homozygous (lit/lit) and heterozygous (lit/+ ) pbARR2-Cre, PTEN(fl/fl) (PTEN – / – ) mice. How suppressed GH/IGF-I levels impacted growth of PTEN – / – mouse-derived prostate cells (MPPK) was examined by growth and survival signaling of cells cultured in lit/+ or lit/lit serum.

RESULTS: Body weight, prostate weight and serum GH and IGF-I levels were reduced in lit/lit relative to lit/+ . PTEN – / – litters. While the anterior lobes of lit/+ + PTEN – / – prostates consistently presented swollen, indicative of ductal blockage, the degree of prostatic dysplasia in 15- and 20-week-old lit/lit and lit/+ + PTEN – / – mice was indistinguishable as measured by normalized prostatic weight, tissue histology, or probasin, PSP94, E-cadherin, N-cadherin and vimentin expression. However, growth and AKT activation of MPPK cells was decreased when cultured in lit/lit serum as compared with lit/+ + serum and restored in lit/lit serum supplemented with IGF-I and, to a lesser extent, GH.

CONCLUSIONS: These results suggest that initiation of prostate carcinogenesis by loss of PTEN is not influenced by germline variation of genes encoding signaling molecules in the GH/IGF-I axis, but suggests that these factors may affect the progression of dysplastic phenotype and supports previous studies, indicating that the GH/IGF milieu does impact the growth of PTEN-deficient dysplastic prostatic cells once transformed.

Keywords: prostatic intraepithelial neoplasia; transgenic mouse; endocrine hormone; phosphatase tensin homolog; growth hormone receptor hormone

INTRODUCTION

The growth hormone and insulin-like growth factor-I (GH/IGF-I) axis is an important regulator of growth, survival and metastatic potential in a variety of malignancies and is strongly implicated in prostate cancer (PCa) etiology and risk.1-4 Perturbations in intrinsic expression of IGF axis components by tumor cells are implicated in susceptibility and progression of PCa.5-12 IGF-I receptor (IGF-1R) expression is elevated in metastatic PCa.13,14 and castration-resistant disease progression is associated with increased expression of IGF-I and IGF-1R.15 Furthermore, maintaining IGF-I responsiveness facilitates PCa survival and growth, and is achieved through androgen-modulated IGF-1R expression.14,16,17

IGF-I is a potent mitogen and antiapoptotic factor predominantly produced by the liver in response to GH signaling.18 Ligand activation of IGF-1R results in the activation of multiple intracellular signaling pathways, including RAS/extracellular signal-regulated kinases (ERKs) 1 and 2 and phosphatidylinositol-3 kinase (PI3K)/AKT that control the various IGF-mediated biological effects.19 Activating mutations of PI3K have also been linked to PCa progression,20 indicating the importance of deregulated PI3K signaling in PCa. The phosphatidylinositol phosphatase tumor suppressor, PTEN, is an important negative regulator of PI3K signaling. Homozygous loss of PTEN is a recurrent event in advanced PCa,21 and among those patients who are not PTEN null, many exhibit loss of one PTEN allele.22 We have previously reported that the GH/IGF-I axis is important for androgen-responsive growth, castration-resistant progression and growth of androgen receptor (AR)-negative, PTEN-deficient PCa xenografts in the lit/lit GH-releasing hormone-R loss-of-function murine host.23 In addition, hemizygous PTEN loss, alone and in combination with the presence of TMPRSS2-ERG gene rearrangements, are reported in early stages of disease and to predict an increased risk of biochemical progression.24,25 This prediction is supported by the demonstration that a murine model for prostate-conditional PTEN loss is sufficient to induce development26 and castration-resistant progression27 of PCa. These results indicate that PTEN

1The Vancouver Prostate Centre, Vancouver General Hospital, Vancouver, British Columbia, Canada; 2Department of Urology, Osaka Medical College, Osaka, Japan; 3Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada; 4Department of Urologic Sciences, University of British Columbia, Vancouver, British Columbia, Canada and 5Department of Medicine and Oncology, McGill University, Montreal, Quebec, Canada. Correspondence: Dr ME Cox, The Vancouver Prostate Centre, Vancouver General Hospital, 2660 Oak Street, Vancouver, British Columbia, Canada V6H 3Z6.

E-mail: mcox@prostatecentre.com

6These authors contributed equally to this work.
loss can hypersensitize cells to PI3K activation by factors such as GH and IGF-I.

To test the hypothesis that dysplastic initiation of PTEN-deficient prostate epithelial cells may be due to hypersensitivity to GH/IGF-mediated PI3K signaling, we assessed development of prostatic dysplasia in Ghrhr Delta60G (lit/+) pRAR2-Cre, PTEN(fli/fli) mice (PTEN−/−). Our in vivo and in vitro results suggest that prostatic dysplasia induced by PTEN loss is not substantially affected by Ghrhr deficiency, but subsequent growth of dysplastic epithelia is impaired in hosts carrying germline deficiencies for GH and IGF-I, expression, and may affect the progression of dysplastic phenotype in PCa.

MATERIALS AND METHODS

Ghrhr(lit/lit) and (lit/+)+ pRAR2-Cre/PTEN(fli/fli) mouse characterization

PTEN(fli/fli) C57BL/6J mice were obtained from T. Mak (Toronto, Canada)28 pRAR2-Cre created in a C57BL/6 × DBA2 hybrid mouse strain was obtained from P. Roy-Berman (Los Angeles, CA)29 and Ghrhr Delta60G (lit/lit) C57BL/6J mice were from Jackson Laboratory (Bar Harbor, ME).30 pRAR2-Cre, PTEN(fli/fli) mice (PTEN−/−) generated, as described previously,31 were crossed with lit/lit mice to produce lit/lit and lit/+ PTEN−/− mice. This transgenic strain was genotyped to confirm the status of the Ghrhr allele mutation as described previously,32 as well as confirming Cre recombination and PTEN(fli/fli) status from tail clip DNA extracted using DirectPCR Lysis Reagent (Viagen Biotech, Los Angeles, CA) and recombinase and PTEN(fl/fl) status from tail clip DNA extracted using a tail clip DNA extraction kit (QIAGEN). The resulting lanced weight of the hypertrophic anterior prostate lobes in both cohorts (Figures 1a and b, respectively). However, the anterior prostate lobes from lit/+ PTEN−/− mice consistently presented as swollen and fluid-filled, while those from lit/lit PTEN−/− mice were enlarged and structurally nodular, with no evidence of fluid retention. Histopathologic analysis of dysplastic prostates from lit/lit and lit/+ PTEN−/− mice (Figures 1c and d, respectively) revealed that both cohorts exhibited uniformly mild-to-severe prostatic epithelial dysplasia in the anterior, ventral and dorsolateral prostatic lobes, with no clear evidence of stromal invasion, consistent with induction of prostatic intraepithelial neoplasia as previously reported in PTEN−/− mice.32

As predicted from the known effects of the Ghrhr Delta60G deficiency of lit/lit mice, the average serum GH level of lit/lit PTEN−/− mice at 15 and 20 weeks (0.2 and 0.6 ng ml−1, respectively) was on average ~1/10th that measured in lit/+ PTEN−/− mice (3.1 and 3.5 ng ml−1, respectively; Figure 2a). Correspondingly, the average serum IGF-I level of lit/lit mice at 15 and 20 weeks (37 and 42 ng ml−1, respectively) was ~1/6th that measured in lit/+ PTEN−/− mice (247 and 267 ng ml−1; Figure 2b). Consistent with our previous characterizations of male lit/lit and lit/+ mice,33 the average body weight of lit/lit PTEN−/− mice (18 and 20 g at 15 and 20 weeks, respectively) was ~60% that of the lit/+ PTEN−/− littermates (31 and 33 g at 15 and 20 weeks, respectively; Figure 2c). To normalize tissue weights, the anterior prostate lobes of the lit/+ PTEN−/− mice were drained of retained fluid. The resulting lanced weight of the prostates from lit/lit PTEN−/− mice at 15 and 20 weeks (0.13 and 0.17 g, respectively) were also proportionally smaller (~60%) than that of their lit/+ littermates (0.19 and 0.25 g, respectively; Figure 2d). We next assessed whether PTEN−/− prostatic dysplasia was distinguishable when normalized for intrinsic differences in body size of lit/lit and lit/+ mice, the prostate:body weight ratio was calculated for each animal and the average body weight-normalized prostate weight ratio was compared between

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Statistical analysis

Data were analyzed by analysis of variance and Mann–Whitney post hoc test, except for paired tests that were analyzed by Student’s t-test. Levels of statistical significance were set at P<0.05.

RESULTS

lit/lit PTEN−/− mice and lit/+ PTEN−/− mice display extensive prostatic dysplasia

To compare the impact of GH/IGF-I axis deficiency on development of prostatic dysplasia induced by loss of PTEN in the murine prostate, 10 lit/lit and lit/+ PTEN−/− mice were collected at 15 and 20 weeks of age. Macromorphologic examination of the prostates from lit/lit PTEN−/− and lit/+ PTEN−/− mice revealed overtly hypertrophic anterior prostate lobes in both cohorts (Figures 1a and b, respectively). However, the anterior prostate lobes from lit/+ PTEN−/− mice consistently presented as swollen and fluid-filled, while those from lit/lit PTEN−/− mice were enlarged and structurally nodular, with no evidence of fluid retention. Histopathologic analysis of dysplastic prostates from lit/lit and lit/+ PTEN−/− mice (Figures 1c and d, respectively) revealed that both cohorts exhibited uniformly mild-to-severe prostatic epithelial dysplasia in the anterior, ventral and dorsolateral prostatic lobes, with no clear evidence of stromal invasion, consistent with induction of prostatic intraepithelial neoplasia as previously reported in PTEN−/− mice.
the lit/lit and lit/+ PTEN −/− cohorts (Figure 2e). Again, the body weight-normalized prostate weight ratio was indistinguishable between the PTEN −/− lit/lit and lit/+ cohorts at 15 or 20 weeks; however, there was a significant increase in the prostate weight increase from 15 to 20 weeks for the lit/+ cohort (60 mg) but not in the lit/lit cohort (30 mg).

Furthermore, immunohistochemical analysis for Ki-67 staining of the dysplastic prostatic epithelia revealed that the mitotic activity between cohorts was indistinguishable (Supplementary Figure S1). Specimens were also assessed for PI3K activity as measured by phospho S473-Akt (pAKT) immunohistochemical staining intensity normalized to AR positivity as a marker of epithelial cell density (Supplementary Figure S2). While we observed high constitutive pAKT levels, as expected, from prostatic PTEN knockout, there was no measurable difference in pAKT levels between cohorts.26,32 We conclude that elevated basal PI3K signaling and mitotic activity in PTEN-deficient prostates does not require normal physiologic IGF-I or GH levels.

In a parallel analysis, we compared prostatic pathology of 15-week-old lit/lit PTEN −/− mice with Ghhr wild-type (wt) PTEN −/− mice (Supplementary Figure S3). The overtly hypertrophic and swollen prostatic phenotype of the Ghhr wt PTEN −/− mice was indistinguishable from that of age-matched lit/+ PTEN −/− mice. The body weight-normalized total prostate weights of the Ghhr wt PTEN −/− mice was 1.5 times greater than that of lit/lit PTEN −/− mice, but again this difference was

**Figure 1.** lit/lit PTEN −/− and lit/+ PTEN −/− mice display extensive prostatic dysplasia. Representative macroscopic images of (a) lit/lit PTEN −/− mouse and (b) lit/+ PTEN −/− mouse prostates show overtly hypertrophic anterior prostate (AP) below seminal vesicles (SV) and urinary bladder. Representative low magnification (upper panels, scale bar = 1 mm) and high magnification (lower panels, scale bar = 100 μm) microscopic images of hematoxylin- and eosin-stained sections of dysplastic mouse AP, dorsal–lateral prostate (DLP) and ventral prostate (VP) from (c) lit/lit PTEN −/− 20 weeks and (d) lit/+ PTEN −/− 20 weeks. PTEN, phosphatase and tensin homolog.
not seen when the Ghrhr wt PTEN −/− prostates were lanced to drain retained fluid. These results indicate that the lack of a difference between the lit/lit and lit/+ prostates was not due to a suppressed effect in response to Ghrhr D60G heterozygosity.

Assessment of epithelial and mesenchymal characteristics in dysplastic prostates of lit/lit and lit/+ PTEN −/− mice

To assess whether GH/IGF-I deficiency impacts prostatic epithelial differentiation in PTEN −/− mice, transcript levels of probasin (Figure 3a) and PSP94 (Figure 3b) were measured by real-time reverse transcription-polymerase chain reaction analysis from total RNA of each mouse prostate collected at 15 and 20 weeks of age. Although the average mean expression of these markers in the lit/lit cohort at 20 weeks trended higher than the average of the other three cohorts, the expression of these prostatic epithelial markers was statistically indistinguishable between the lit/lit and lit/+ cohorts at either time point or between time points for each cohort.

RAS/ERK pathway activation has been reported to promote epithelial-to-mesenchymal transition (EMT) in the prostates of PTEN −/− mice.33 To assess whether the slightly lower average probasin and PSP94 expression in the 20-week lit/+ cohort was associated with any evidence of EMT, real-time polymerase chain reaction was used to measure transcript levels of E-cadherin (Figure 3c), vimentin (Figure 3d) and N-cadherin (Figure 3e) from total RNA of each mouse prostate collected above. Normalized E-cadherin expression level was indistinguishable between the cohorts at either time point; however, there was an apparent increase in the relative expression of vimentin and N-cadherin transcripts from 15 to 20 weeks in both the lit/lit and lit/+ cohorts. Although prostatic N-cadherin levels appeared to increase between 15 and 20 weeks in lit/+ PTEN −/− mice compared with the lit/lit PTEN −/− mice, this difference was dominated by an apparent suppression of N-cadherin expression in the lit/+ cohort that is likely a sampling artifact, as the mean N-cadherin expression level of the 15-week lit/lit and lit/+ PTEN −/− cohorts was statistically indistinguishable. However, when protein expression for these markers was assessed from

Figure 2. Comparison of lit/lit and lit/+ PTEN −/− male mouse body weight, serum growth hormone (GH) levels and serum insulin-like growth factor-1 (IGF-I) levels. Average (± s.e.m.) serum GH (a) and IGF-1 (b) levels, and body weight (c) and lanced prostate weight (d) of lit/lit (black bars) and lit/+ (white bars) PTEN −/− mice at 15 and 20 weeks of age. (e) Prostate:body weight ratio was calculated for each animal and the average prostate/body weight ratio (± s.e.m.) was plotted for lit/lit (black bars) and lit/+ (white bars) PTEN −/− mice. *P<0.05 by analysis of variance and Mann–Whitney post hoc test; n.s., no significant difference observed for indicated comparisons. PTEN, phosphatase and tensin homolog.
prostatic lysates by immunoblotting normalized to β-actin levels, these trends for increased vimentin and N-cadherin expression in the 20-week cohorts were not confirmed (Supplementary Figure S4).

We conclude that although the consistently observed swelling of the anterior prostate lobes in the lit/+ PTEN−/− mouse, indicative of blocked terminal prostatic ducts in these animals, suggests that dysplastic progression is delayed in the lit/lit hosts, morphologic and molecular marker expression changes indicate that genomic alterations that result in suppressed GH/IGF-I axis signaling do not affect initiation of prostatic dysplasia because of PTEN deletion.

Characterization of EMT marker expression in MPPK cells
We next assessed whether the previously established PTEN−/− mouse prostate cell line, MPPK, harbored any of the EMT characteristics implicated above by characterizing them for expression of prostatic epithelial and mesenchymal markers relative to that observed in well-characterized, androgen-responsive human prostate cancer cell line, LNCaP, and androgen-independent, AR-negative human prostate cancer cell line, DU145 (Figure 4a). MPPK cells expressed AR and this expression was enhanced in the presence of R1881. Androgen responsiveness was confirmed by firefly luciferase reporter assay in MPPK cells transfected with pbARR2-Luc and stimulated 24 h with R1881 (Figure 4b). MPPK cells were also confirmed to be deficient for PTEN expression by comparison to expression in the PTEN wt DU145 cells and the PTEN-null LNCaP cells. MPPK cells exhibited robust E-cadherin expression, comparable to that observed in LNCaP cells. While MPPK cells expressed vimentin at low levels, comparable to that of LNCaP cells, relative to the robust expression in DU145 cells, they did express high levels of N-cadherin not observed in LNCaP and DU145 cells. This observation is consistent with the suggestion that PTEN−/− prostatic epithelial cells acquire intermediary mesenchymal characteristics while still in an intraepithelial neoplastic state and indicate that the MPPK cell line accurately reflects the phenotype of the dysplastic prostatic epithelia of PTEN−/− cells.

Growth of MPPK cells is suppressed in media supplemented with lit/lit serum
To determine if growth characteristics of MPPK cells were sensitive to changes in GH/IGF-I levels, relative proliferation of MPPK cells cultured in basal media + 1% lit/lit serum or 1% lit/+ serum was
examined by BrdU incorporation (Figure 4c). Mitotic activity of MPPK cells cultured in 1% lit/+ serum was significantly elevated (~40%) relative to that of cells cultured in 1% lit/lit serum. RAS/mitogen-activated protein kinase and PI3K/AKT signaling have been implicated in transformation of PTEN−/− murine prostate epithelia. The effect of these culture conditions on steady-state RAS/mitogen-activated protein kinase and PI3K/AKT signaling was assessed by immunoblotting of MPPK, whole-cell lysates after culture in 1% lit/lit serum or 1% lit/+ serum for 2 days for phospho- and total AKT and ERK, and vinculin (Figure 4d). Densitometric quantification of phospho- to total AKT and Erk revealed that steady-state phospho-AKT levels were on average elevated fivefold, while phospho-ERK levels were not affected in cells cultured in lit/+ serum vs those cultured in lit/lit serum. These observations are consistent with enhanced PI3K/AKT signaling in PTEN-deficient cells when cultured in the presence of GH- and IGF-I-containing serum.

Suppressed growth of MPPK cells in lit/lit serum can be rescued by the addition of GH or IGF-I

To determine whether GH or IGF-I were sufficient to promote growth in lit/lit serum, MPPK cells were cultured in 1% lit/lit serum ± recombinant GH (0.1, 1 and 10 ng ml⁻¹) or recombinant IGF-I (5, 50 and 100 ng ml⁻¹) or 1% lit/+ serum for 2 days (Figure 5a). Relative mitotic activity was measured by BrdU incorporation and expressed relative to that of cells cultured without the addition of exogenous growth factors. The addition of GH to levels equal to or greater than that found in lit/+ serum marginally affected MPPK cell proliferation, while supplementing lit/lit serum with IGF-I significantly enhanced MPPK proliferation at all concentrations tested. In contrast, proliferation of MPPK cells cultured in GH was significantly lower than that of cells grown in lit/+ serum, while proliferation of cells grown in IGF-I were indistinguishable from that of cells grown in lit/+ serum. These results suggest that suppressed IGF-I levels are primarily responsible for lower proliferation rate of MPPK cells in vitro.

To assess if intracellular signaling pathways were altered by growth factor supplementation of the lit/lit serum, lysates of MPPK cells cultured 24 h in serum-free media and stimulated with 1% lit/lit serum plus 0, 0.1 and 1 ng ml⁻¹ GH, or 0, 5 and 50 ng ml⁻¹ IGF-I for 20 min were immunoblotted against phospho- and total AKT and ERK, and vinculin (Figure 5b). Paralleling the observed changes in cell growth, GH did not affect steady-state AKT or ERK activation at the 20 min time-point, while IGF-I did promote AKT activation. These results are consistent with the differences observed in proliferation and AKT activation in MPPK cells cultured in lit/lit vs lit/+ serum and suggests that IGF-I can rescue suppressed proliferation of these cells in serum from lit/lit mice.

DISCUSSION

The GH/IGF-I axis and downstream signaling pathways, such as RAS/ERK and PI3K/AKT, have established roles in PCa patho-
In contrast, development of TRAMP tumors in hosts harboring targeted deletion of hepatic IGF-I did not show a significant phenotypic difference in prostatic dysplastic growth in mice with PTEN deficiency due to PTEN loss. While this PTEN deficiency was associated with a decrease in epithelial hyperplasia in this animal model, it did not affect the expression of epithelial markers such as prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA).

In vitro proliferation and activation of AKT and ERK1/2 signaling in MPPK cells cultured in recombinant growth hormone (GH) and insulin-like growth factor-1 (IGF-I). MPPK cells were cultured in 1% GH and IGF-I levels are important regulators of PTEN-deficient prostates. Recently, AKT/ERK activation was reported to cooperate with PTEN deficiency to promote EMT and metastatic behavior of prostate progenitor cells. We therefore assessed epithelial and mesenchymal characteristics of these cohorts. Murine prostate epithelial differentiation marker expression (probasin, PSP94 and E-cadherin) were indistinguishable in the two time points analyzed here. The expression of mesenchymal markers, vimentin and N-cadherin, showed no significant difference between our experiments. This lack of overt phenotypic difference in prostatic dysplastic formation compelled us to examine molecular features of the PTEN deficiency using various human cancer lines as xenografts in immune-compromised mice, including inhibiting growth of PC3 and DU145 PCa.
We have previously demonstrated that decreased circulating GH and IGF-I were significant contributors to suppressed growth of both androgen-responsive and castration-resistant PTEN-null PCa cells, and that antisense oligonucleotide suppression of IGF-1R expression suppressed growth of these androgen-responsive and castration-resistant PTEN-null PCa cells. These studies supported the contention that decreased IGF-I availability and signaling from IGF-1R to AKT was the primary missing growth stimulatory pathway for such cells in the \( \text{lit/lit} \) host. Consistent with these previous results, in this study, growth of MPPK cells was suppressed in media supplemented with \( \text{lit/lit} \) serum as compared with \( \text{lit/+} \), serum, and that restoration of suppressed growth of MPPK cells in \( \text{lit/lit} \) serum by the addition of IGF-I and, to a lesser extent, GH was correlated with increased steady-state activation of AKT. In contrast to the results from TRAMP crosses with \( \text{lit/lit} \) or hepatic IGF-I knockout mice that implicated a role for GH in driving TRAMP tumorigenesis, these \textit{in vitro} experiments and our previous studies implicate circulating IGF-I as the predominant contributor to proliferation of PCa, and that this effect is mediated primarily through PI3K/AKT activation.

CONCLUSION

Our \textit{in vivo} and \textit{in vitro} experiments suggest that loss of PTEN may initiate prostatic epithelial dysplasia and acquisition of mesenchymal characteristics and that IGF-I may be an important factor in sustaining proliferation of these dysplastic cells. We therefore suggest that in early stages of PCa development, suppression of GH/IGF-I axis may delay invasive PCa progression. Such treatments might be best augmented by therapies targeting factors that regulate EMT, a process mechanistically linked with stem cell signatures in PCa cells and increased resistance to apoptosis, diminished senescence, escape from immune surveillance and eventual resistance to therapy. With the reports linking GH/IGF-I axis signaling to EMT and acquisition of stem-like properties, these results support continued effort to disrupt IGF axis signaling to control PCa progression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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