Androgen Receptor Upregulation Mediates Radioresistance after Ionizing Radiation

Daniel E. Spratt1,2,3, Michael J. Evans2, Brian J. Davis4, Michael G. Doran3, Man Xia Lee2, Neel Shah2, John Wongvipat2, Kathryn E. Carnazza3, George G. Klee5, William Polkinghorn1,2, Donald J. Tindall5, Jason S. Lewis3, and Charles L. Sawyers2

Abstract

Clinical trials have established the benefit of androgen deprivation therapy (ADT) combined with radiotherapy in prostate cancer. ADT sensitizes prostate cancer to radiotherapy-induced death at least in part through inhibition of DNA repair machinery, but for unknown reasons, adjuvant ADT provides further survival benefits. Here, we show that androgen receptor (AR) expression and activity are durably upregulated following radiotherapy in multiple human prostate cancer models in vitro and in vivo. Moreover, the degree of AR upregulation correlates with survival in vitro and time to tumor progression in animal models. We also provide evidence of AR pathway upregulation, measured by a rise in serum levels of AR-regulated hK2 protein, in nearly 20% of patients after radiotherapy. Furthermore, these men were three-fold more likely to experience subsequent biochemical failure. Collectively, these data demonstrate that radiotherapy can upregulate AR signaling after therapy to an extent that negatively affects disease progression and/or survival. Cancer Res; 75(22): 4688–96. © 2015 AACR.

Introduction

Several phase III clinical trials have demonstrated a clear survival benefit when long-term adjuvant androgen deprivation therapy (LTADT) is added to concurrent ADT and external beam radiotherapy (1, 2). The addition of adjuvant ADT after radiotherapy is commonly referred to as LTADT; however, recent evidence suggests that simply increasing duration of ADT without the focus on when it is given in relation to radiotherapy does not improve outcomes, suggesting the timing and duration of ADT are critical (3). However, chronic androgen suppression can impact quality of life, motivating ongoing clinical studies to optimize the duration of androgen deprivation without compromising efficacy (3, 4).

We previously have shown that primary prostate tumors display heterogeneity in androgen receptor (AR) transcriptional output, which could result in differential sensitivity to ADT and to the relative clinical benefit of ADT when combined with radiotherapy (5). Furthermore, we and others have also shown that AR activates DNA repair pathways, providing further rationale for concurrent ADT/radiotherapy (5–7). Despite this radiosensitizing mechanistic action of ADT, clinical trials have demonstrated that adjuvant ADT has similar efficacy to that of concurrent ADT with radiotherapy (8), begging the question why adjuvant ADT sufficiently compensates for radiosensitizing concurrent therapy, and further improves survival even beyond concurrent use of ADT with radiotherapy (1).

In addition to variability in baseline AR signaling, select observations may suggest that AR signaling is upregulated by radiotherapy. For instance, in small patient series, a subset of patients have increases in secreted levels of AR target genes (e.g., PSA, hK2) during external beam radiotherapy (EBRT; refs. 9, 10). In addition, the AR target gene TMPRSS2 is upregulated in a human prostate cancer cell line exposed to therapeutic doses of radiotherapy (6). These considerations led us to more broadly study the impact of radiotherapy on AR signaling, and the association between high AR signaling after radiotherapy and measures of outcome, as this may have implications for the use and duration of adjuvant ADT after radiotherapy.

Materials and Methods

Cell lines

LNCaP and MDA-PCa2b cell lines used were purchased directly from the American Type Culture Collection and cultured according to recommended specifications. LNCaP-AR is an AR-overexpressing (wild-type) cell line originally derived from parental LNCaP with a luciferase probasin reporter. LNCaP-AR cell line was authenticated via AR overexpression by PCR, immunoblot, and luciferase assay. CWR22Pc were obtained from Marja Nevailanan, Thomas Jefferson University.
Cell culture

Cell lines were not kept in culture longer than 6 months. Growth conditions for each cell line are described in Supplementary Methods.

Cell irradiation

All described doses of radiation were delivered using Cesium-137 irradiator (Shepherd Mark, Model 65). Correction factors for decay were implemented, and the estimated dose rate of delivered was 184 cGy/min. All plates were continuously rotated with a turntable speed of 6 revolutions/minute to improve dose homogeneity.

Real-time PCR

Cells were plated and 24 hours later irradiated. At the specified time after radiotherapy, cells were collected for RNA extraction using the Qiagen Kit and RNA-easy Kit (QIAGEN, QIA Shredder, #79656, QIAGEN, RNasey Mini Kit, #74106). cDNA was generated using the Applied Biosystems High capacity cDNA Reverse Transcription Kit (#4368814). Per manufacturer’s recommendations, Quantifast (QIAGEN, Quantifast SYBR Green PCR Kit, #204057) was used for PCR. All assays were performed in quadruplicate and normalized to actin. PCR primers can be found in Supplementary Methods.

Western blot analysis

Whole-cell lysates were prepared using 10% M-PER lysis buffer and clarified by centrifugation. Proteins were separated by 4% to 12% SDS-PAGE 15-well gel prepared as previously described. Primary antibodies for the following proteins were used: AR [Santa Cruz; AR (N-20), sc-816, 1:500 dilution], γ-H2AX [Millipore; Anti-phospho-Histone H2AX (ser139), clone JBW301, 05–636, 1:500–1,000 dilution], pChk2 [Cell Signaling Technology; p-Chk2 (T68) (C13C1), 2197s, 1:1,000 dilution], and GAPDH [Abcam; GAPDH, ab9485 (1:10,000)]. Secondary antibodies used included Jackson Immuno Research, Goat anti-mouse HRP (115-035-003, 1:10,000 dilution) or gamma-H2aX (Invitrogen; Alexa Fluor 488, A11001, 1:500 dilution) for AR (Vector Laboratories; Dylight 594, D1-1594, 1:100 dilution) or gamma-H2aX (Invitrogen; Alexa Fluor 488, A11001, 1:500 dilution) of AR function was determined by measuring luciferase activity of human LNCaP-AR xenografts grown in male mice. These tumors coexpressed exogenous AR and the AR-dependent luciferase promoter cassette.

Immunofluorescence assays

LNCaP and LNCaP-AR cells were grown as described above in parallel for 24 hours on 4-chamber slides (Thermo Scientific, Lab-Tek II Chamber Slide w/cover CC2 Glass slide sterile, 154917), approximately 125,000 cells/well in 500 μL total volume. Following radiotherapy using the cell irradiator, cells were washed twice with PBS and fixed with 4% PFA and 0.2% Triton X-100. The primary antibody for AR [Santa Cruz; AR (N-20), sc-816, 1:200 dilution] or gamma-H2aX (Millipore; Anti-phospho-Histone H2aX (ser139), clone JBW301, 05–636, 1:200 dilution) incubated overnight at 4 degrees, and then washed followed by incubation with the secondary antibody for AR (Vector Laboratories; Dylight 594, D1-1594, 1:100 dilution) or gamma-H2aX (Invitrogen; Alexa Fluor 488, A11001, 1:500 dilution) for 1 hour at room temperature, and then washed followed by incubation with the secondary antibody for AR (Vector Laboratories; Dylight 594, D1-1594, 1:100 dilution) or gamma-H2aX (Invitrogen; Alexa Fluor 488, A11001, 1:500 dilution) of AR function was determined by measuring luciferase activity of human LNCaP-AR xenografts grown in male mice. These tumors coexpressed exogenous AR and the AR-dependent luciferase promoter cassette.
radical prostatectomy. Serum specimens were collected, analyzed, and stored at baseline before the initiation of radiotherapy and at the first follow-up visit after treatment (3–7 months after radiotherapy). Both serum PSA and free PSA were measured utilizing the Hybritech assays on an Access analyzer (Beckman Coulter, Inc). Free-hK2 levels were measured utilizing a selective pair of monoclonal antibodies. The assay was implemented on the Access analyzer, and the cross-reactivity with PSA is negligible as previously described (11). The hK2 limit of detection was 1.5 pg/mL, and the day-to-day coefficient of variation set at <15% was <4 pg/mL.

Statistical analyses
All qPCR analyses, IHC comparisons, and comet assay comparisons were performed with a two-sided t test. Correlation statistics were performed using R² statistics. In vivo tumor growth comparisons were estimated by actuarial likelihood estimates using the inverse Kaplan–Meier method with log-rank statistics to obtain cumulative incidence rates. Statistical analysis was performed using SPSS version 21 (SPSS, Inc.). Patient data statistics were conducted using logistical regression to compare patients with and without a rise in hK2 post-radiotherapy, reported as an OR and 95% confidence interval (CI). Kaplan–Meier time point comparisons (72 months) were performed with a c² test. Baseline patient group comparison between those with and without hK2 gain was performed using the Fisher exact test or Mantel–Haenszel c² test for ordinal and categorical variables and the Wilcoxon–Mann–Whitney test for continuous variables. For all analyses, two-sided P values of ≤ 0.05 were considered statistically significant.

Results
We first tested whether radiotherapy had direct biologic effects on AR regulation and expression. Four human prostate cancer cell lines (LNCaP, LNCaP-AR, MDA-PCa2b, and CWR22Pc) showed a dose-dependent increase in AR mRNA after exposure to 1, 6, and 12 Gy at 24 hours after radiotherapy (Fig. 1A). AR protein levels were also upregulated in three (LNCaP, LNCaP-AR, and CWR22Pc) of the four cell lines at the same time points (Fig. 1B). AR nuclear protein translocation was also enhanced by radiotherapy, as immunofluorescence showed that the overall percentage of nuclear AR, measured 24 hours after radiotherapy, was 25%- to 45%-fold higher compared with vehicle control (Supplementary Fig. S1A). To provide further evidence of AR protein upregulation, we used a ligand binding assay with 18F-labeled dihydrotestosterone (18F-FDHT; Supplementary Fig. S1B). When 18F-FDHT was added to LNCaP cells plated in androgen-free media and irradiated with increasing doses of radiotherapy, even at 1 Gy, there was a significant increase in uptake of 18F-FDHT. This increase is consistent with increased AR protein expression and/or available ligand binding sites, and was abrogated when the experiment was performed in androgen-replete serum (due to competition of unlabeled ligand with 18F-FDHT).

To test if radiotherapy-induced AR upregulation occurs in vivo, subcutaneous LNCaP-AR tumors were established in mice and treated with 10 Gy of conformal external beam radiotherapy, and
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AR mRNA after EBRT (Fig. 3A). Of note, the magnitude of AR relativity low baseline AR expression but large induction of AR among cell lines as larger changes in radiosensitivity as well as AR output. AR induction across the cohort, with 19 of the 25 mice (76%) being able to measure AR pathway activation also revealed heterogeneity in to tumor shrinkage after treatment. Because a considerable fraction of prostate cancer cells die during radiotherapy, one would expect overall serum hK2 levels would decline after radiotherapy. Thus, an absolute rise in hK2 after therapy could represent a large increase in hK2 protein expression on a per cell basis.

Discussion

For decades the understanding of why the addition of ADT to radiotherapy improves survival has remained elusive (12–14).
This question was further complicated by the knowledge that the addition of ADT to radical prostatectomy failed to improve survival (15). The recent discovery that ADT inhibits nonhomologous end joining, a critical DNA repair process, provides a compelling answer to years of observed clinical trial outcomes (5–7). However, the new found clarity on this topic has subsequently created new questions. For instance, multiple randomized trials have demonstrated that the use of adjuvant ADT after combined ADT/radiotherapy also improves survival, but by unknown mechanisms (1,14). In contrast, a recently reported phase III trial tested whether an increase in the duration of ADT prior to the start of radiotherapy would improve outcome, but failed to demonstrate a benefit for prolonged neoadjuvant ADT (3). These results suggest the timing of ADT in relationship to radiotherapy is perhaps more critical than simply the duration of use.

In the present report, we address the related issue of adjuvant ADT and provide mechanistic insight into why this clinical practice may be beneficial. Specifically, we show that radiotherapy induces upregulation of AR expression and activity across a panel of human prostate cancer cell lines, and the magnitude of this upregulation is more strongly correlated with increased viability in a clonogenic survival assay than baseline AR expression. This association was confirmed in xenograft experiments, where LNCaP-AR tumors with the greatest percent increase in AR signaling after radiotherapy showed more rapid time to progression. These preclinical findings appear to be relevant in patients,
because we found that men experiencing an increase in serum hK2 levels after radiotherapy were 3 times more likely to experience a biochemical failure than those with unchanged or declining hK2 after treatment. Finally, we demonstrate that baseline AR and AR output do not correlate with tumor response in vitro, in vivo, or in our patient serum data of hK2 levels.

Prior studies have reported that approximately 20% of men have increases in PSA during EBRT treatment (without the use of ADT), whereas the remainder had negatively sloping PSA declines (9). These results have previously been ignored due to the absence of evidence for clinical significance of inferior treatment outcomes (16). This clinical heterogeneity
is also seen in our preclinical studies. One hypothesis that might explain the heterogeneity is variable amounts of hypoxia present in tumors, particularly since ADT has been shown to reduce prostate cancer hypoxia, suggesting an interplay with AR signaling (17, 18). Our results suggest that AR activity during and after ADT/EBRT should be more closely studied with serum and imaging biomarkers to determine their prognostic significance. One potential implication is that adjuvant ADT may only be necessary for those men whose tumors upregulate AR as a response to radiotherapy. Alternatively, more potent AR inhibition using second-generation ADT might prevent or mitigate the negative consequences of AR upregulation after radiotherapy.

The underlying biologic mechanism by which EBRT upregulates AR expression remains to be defined. Because AR mRNA levels are elevated in a dose-dependent manner by EBRT, it is possible that a transcription factor that is sensitive to genotoxic stress elevates AR transcription. Potential candidates include Ku70 and Ku80, NF-κB, and the STAT family (19–21). Ku is particularly promising given that the Ku70/Ku80 heterodimer recruits DNA-PKcs to double DNA strand breaks, and it has been demonstrated that both Ku70 and Ku80 directly interact with the ligand binding domain of the AR (21, 22). In addition, the heterogeneity in magnitude and kinetics of radiotherapy-induced AR upregulation seen across in vitro and in vivo experiments is consistent with a stochastic variable that affects radiotherapy response or a preexisting subset of tumor cells primed to respond to radiotherapy in this manner as an adaptive resistance mechanism. These critical points are areas for future investigation.

Disclosure of Potential Conflicts of Interest
B.J. Davis reports receiving commercial research grant from Takeda UK LLC; has received speakers bureau honoraria from ASTRO, DMCI Inc., and Prospect Medical Inc.; has ownership interest (including patents) in Pfizer; and is a consultant/advisory board member for EDAP Technomed Inc. J. Wongvipat has ownership interest (including patents) in patents for Enzalutamide/MDV3100/Xtandi. G.G. Klee reports receiving commercial research support from and has ownership interest (including patents) in Beckman Coulter. W. Polkinghorn is CEO at and has ownership interest (including patents) in Driver Group. D.J. Tindall reports receiving commercial research grant from Beckman Coulter. C.L. Sawyers has provided expert testimony for co-inventor of MDV3100 (enzalutamide) and is entitled to royalties. No potential conflicts of interest were disclosed by the other authors.

Figure 4.
Serum hK2 upregulation after radiotherapy is associated with biochemical failure in 227 men treated with definitive EBRT for localized prostate cancer. A, waterfall plot of percent change in hK2 after radiotherapy compared with baseline. B, comparison of biochemical relapse outcomes in men who had an hK2 gain after radiotherapy (n = 40) versus those who did not (n = 187); (ORs 3.39; 95% CI, 1.23–9.39; P = 0.019). C, Kaplan–Meier curves for freedom of PSA progression by hK2 gain status. Estimates at 72 months for hK2 gain were 83.5% compared with 94.2% for those without an hK2 gain (P = 0.027).
Table 1. Baseline characteristics of patients by hK2 gain status

<table>
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<th>Characteristic</th>
<th>Total (N = 227)</th>
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<th>hK2 increase (N = 40)</th>
<th>P value</th>
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<td>Median</td>
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<td>7 (3.7%)</td>
<td>2 (5.0%)</td>
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aFisher exact test.  
bWilcoxon-Mann-Whitney test.  
cMantel-Haenszel \( \chi^2 \) test.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.E. Spratt, B.J. Davis, M.G. Doran, M.X. Lee, G.G. Klee

Study supervision: B.J. Davis, J.S. Lewis, C.L. Sawyers

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