A Mechanism for Androgen Receptor-mediated Prostate Cancer Recurrence after Androgen Deprivation Therapy

Christopher W. Gregory, Bin He, Raymond T. Johnson, O. Harris Ford, James L. Mohler, Frank S. French, and Elizabeth M. Wilson


Abstract

The development and growth of prostate cancer depends on the androgen receptor and its high-affinity binding of dihydrotestosterone, which derives from testosterone. Most prostate tumors regress after therapy to prevent testosterone production by the testes, but the tumors eventually recur and cause death. A critical question is whether the androgen receptor mediates recurrent tumor growth after androgen deprivation therapy. Here we report that a majority of recurrent prostate cancers express high levels of the androgen receptor and two nuclear receptor coactivators, transcriptional intermediary factor 2 and steroid receptor coactivator 1. Overexpression of these coactivators increases androgen receptor trans-activation at physiological concentrations of adrenal androgen. Furthermore, we provide a molecular basis for this activation and suggest a general mechanism for recurrent prostate cancer growth.

Introduction

Prostate cancer is a major cause of cancer-related deaths in American men. In its initial stages, prostate cancer is responsive to androgen deprivation therapy achieved by surgical or medical castration, reflecting a dependence on androgen in tumor cell proliferation in early stage disease. However, subsequent to androgen deprivation therapy, prostate cancers recur and progress to a terminal stage despite reduced circulating testosterone. The AR, a member of the steroid receptor family that is activated by testicular androgens, is the major regulatory transcription factor in normal prostate growth and development and in the growth of androgen-dependent prostate cancer. The AR may also contribute to prostate cancer growth during its recurrence in the androgen-deprived patient. A role for AR-mediated gene activation in recurrent prostate cancer is supported by its expression (1, 2) together with the expression of androgen-regulated genes (3). Possible mechanisms for AR reactivation in recurrent prostate cancer include altered growth factor-induced phosphorylation (4–8) and AR mutations (9) that broaden ligand specificity (10). AR gene amplification was observed after androgen depriva-

tion in 30% of recurrent prostate cancers (11). AR overexpression is associated with increased sensitivity to the growth-stimulating effects of low androgen concentrations in recurrent prostate cancer-derived cell lines (12, 13).

Materials and Methods

Immunohistochemistry. Prostate specimens were acquired in compliance with the guidelines of the University of North Carolina at Chapel Hill Clinical Cancer Protocol Review Committee and Institutional Review Board (Chapel Hill, NC). Histological diagnoses were verified by examination of frozen and corresponding formalin-fixed, paraffin-embedded tissues. Samples of BPH from eight men [mean age, 65 (range 58–71) years] and androgen-dependent prostate cancer from eight men [mean age, 59 (range 49–71) years] were obtained from the transition zone and palpable tumor nodules, respectively, in radical prostatectomy specimens. Gleason sum grade was 6 in six men and 7 in two men, and mean serum prostate-specific antigen was 26 (range, 3.5–93) ng/ml. Recurrent prostate cancer was obtained by transurethral resection in eight men who developed urinary retention from a local recurrence of prostate cancer 57 months (mean; range 12–162 months) after androgen deprivation by surgical (four men) or medical (four men) castration. Recurrent tumors were poorly differentiated (Gleason sum, 8 in two men, 9 in four men, and 10 in two men) and serum prostate-specific antigen was 97 ng/ml (mean; range, 0.3–177 ng/ml) despite low serum testosterone (<70 ng/ml) in all of the men. AR gene coding sequence for the prostate cancer samples was wild-type based on denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis for the entire sequence and direct sequencing of the ligand binding domain (14).

Formalin-fixed, paraffin-embedded sections of BPH and androgen-dependent and recurrent human prostate cancer and CWR22 human tumors propagated in nude mice were antigen-reactive by heating at 100°C for 30 min in a vegetable steamer using CITRA or AR 10 buffer (BioGenex Laboratories, San Ramon, CA) and cooled for 10 min. Slides were preincubated with 2% normal horse serum for 5 min at 37°C and washed with automation buffer (Fisher Scientific International, Inc., Pittsburgh, PA). Slides were incubated with antimouse monoclonal antibodies for TIF2, SRC1, and amplified in breast cancer-1 (Transduction Laboratories, Lexington, KY) or AR (BioGenex Laboratories) at 1:300 dilutions followed by incubation with antimouse peroxidase-linked secondary antibody at 1:200 dilution. Immunoperoxidase reaction products were detected using diaminobenzidine.

Immunoblot Analysis. Frozen patient samples and CWR22 tumors (50–100 µg) were pulverized in liquid nitrogen, thawed on ice, and mixed with 1 ml of radiolabeled protein precipitant buffer containing protease inhibitors [0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 50 mM Tris (pH 7.4), 0.5 mM phenylmethylsulfonyl fluoride, 10 µM pepstatin, 4 µM aprotinin, 80 mg/ml leupeptin, 0.2 mM sodium vanadate, and 5 mM benzamidine] and 1 µM DHT. Tissue was homogenized for 20 min, incubated for 30 min on ice, and centrifuged at 10,000 x g for 20 min twice. Supernatant proteins (50 µg) were electrophoresed on 12% acrylamide gels containing SDS and electroblotted to Immobilon-P membranes (Millipore Corp., Bedford, MA). Immunoblot analysis was performed (3) using the antibodies described above and mouse monoclonal anti-progesterone receptor (Affinity Bioreagents, Golden, CO) and rabbit polyclonal anti-estrogen receptor α (Affinity Bioreagents).

Transcription Assays. AR transcriptional assays were performed in the presence or absence of TIF2 overexpression by measuring luciferase activity in...
CV1 cells cotransfected with pCMVhAR (100 ng), wild-type or mutant pCMVhAR507–919 (50 ng), pSG5TIF2 (3 μg), and mouse mammary tumor virus-luciferase reporter vector (5 μg) using calcium phosphate precipitation (15). The absence of the metabolism of androstenedione to testosterone was verified by high-pressure liquid chromatography of CV1 cell extracts.

**GST Adsorption Studies.** GST adsorption incubations were performed (16) using in vitro-translated 35S-labeled AR ligand binding domain (amino acid residues 624–919) and bacterial-expressed GST fusion proteins GST-AR1-333 and GST-TIF2 fusion protein containing TIF2 amino acid residues 1143–1464 designated GST-TIF2-M in the presence of 0.2 μM steroids. Equivalent amounts of GST-AR1-333 and GST-TIF2 protein (~2 μg based on Coomassie Blue staining) were loaded on the gels. Approximately 4 μg of the GST-0 control protein was loaded to ensure the absence of nonspecific binding. Autoradiographic signal intensities in the absence of steroid were essentially identical to the GST-0 control that lacked the AR or TIF2 sequence, as shown previously (15, 16).

**Results and Discussion**

We investigated the role of AR and three nuclear coactivators in recurrent prostate cancer by comparing expression levels in tissue specimens of BPH, androgen-dependent prostate cancer, and recurrent prostate cancer after androgen deprivation therapy. Immunoperoxidase staining of TIF2 was more intense in the nuclei of recurrent prostate cancer than in androgen-dependent prostate cancer or BPH (Fig. 1, b–d). BPH served as the control for prostate cancer because it is more common than normal prostate tissue in men ≥45 years of age. Tissue morphology showed the characteristic glandular structure of BPH, the small, closely spaced glands of early androgen-dependent prostate cancer, and the poor differentiation of recurrent prostate cancer (Fig. 1). Immunostaining for SRC1 was also more intense in recurrent prostate cancer than in BPH or androgen-dependent prostate cancer (Fig. 1, f–h). In contrast, no immunostaining was detected for the nuclear receptor coactivator amplified in breast cancer-1 (data not shown). Immunostaining also indicated high AR expression in recurrent prostate cancer (Fig. 1, j–l), and this was supported by immunoblot analysis (Fig. 2, a–c). Immunoblots confirmed the striking overexpression of TIF2 in six of eight recurrent cancers (Fig. 2c) relative to its barely detectable levels in eight specimens of BPH (Fig. 2a) and

![Fig. 1. TIF2, SRC1, and AR immunoperoxidase staining in human prostate tissue. a, e, and i, androgen-dependent prostate cancer negative controls lacking primary antibody. b, f, and j, BPH tissue. c, g, and k, androgen-dependent (AD) prostate cancer (CaP). d, h, and l, recurrent CaP. ×200.](cancerres.aacrjournals.org)
eight androgen-dependent prostate cancer tissue lysates (Fig. 2b). SRC1 overexpression was evident by immunoblotting in five of eight recurrent cancers (Fig. 2c). Thus, a majority of recurrent cancers had high levels of TIF2 and/or SRC1. The increase in TIF2 in recurrent prostate cancer was greater than that of SRC1, inasmuch as SRC1 was also detected in BPH and androgen-dependent prostate cancer. In control immunoblots, we did not detect progesterone receptor α or β in prostate tissue specimens, but estrogen receptor α was detected and no major difference noted between BPH and androgen-dependent or recurrent prostate cancer (data not shown).

We analyzed TIF2 and SRC1 expression in the CWR22 human prostate cancer xenograft that retains the growth characteristics of human prostate cancers. CWR22 prostate tumors regress after androgen deprivation but recur after several months in the absence of testicular androgens (17, 18). Immunoblots showed decreasing TIF2 levels from 6 to 64 days after androgen deprivation (Fig. 2d). TIF2 expression increased at 98 and 120 days after castration, which coincided with the onset of recurrent prostate cancer cell proliferation and paralleled increased AR expression in this model. SRC1 was also increased in the recurrent CWR22 tumor (Fig. 2d). The results demonstrate that the levels of TIF2 and SRC1 increase with AR expression during the recurrent growth of prostate cancer cells after androgen deprivation.

Transient overexpression of TIF2 in cotransfection assays increased AR-mediated transactivation of a mouse mammary tumor virus-luciferase reporter gene in the presence of different steroids (Fig. 3a). At concentrations of $10^{-9}$ M, androstenedione, estradiol, and progesterone became potent activators of AR in the presence of TIF2. This concentration of androstenedione is within the physiological range of adrenal androgen in the peripheral blood of human males. Transactivation induced by $10^{-7}$ m DHEA was also increased by TIF2 expression.

The AR DNA and ligand binding domain fragment AR507–919 lacks substantial androgen-dependent transcriptional activity in the presence of DHT or other steroids tested in the absence of overexpressed TIF2 (Fig. 3b and data not shown). But with the coexpression of TIF2, AR507–919 is strongly activated by DHT or testosterone.
DHEA, estradiol, androstenedione, and progesterone promoted an

cruitment of TIF2 was confirmed by GST affinity matrix assays.

The ability of lower-affinity steroids to induce wild-type AR re-
cruitment of TIF2 was confirmed by GST affinity matrix assays.

Fig. 4. Steroid dependence of TIF2 interaction with AR. GST affinity matrix assays
of AR ligand binding domain interactions with a TIF2 fragment and AR NH2-terminal
fragment AR507–919 coexpressed with TIF2 required 0.1 nM DHT, 1

ness of AR activation by adrenal androgens that may circulate at
sufficiently high concentrations to recruit highly expressed coactiva-
tors. Under normal physiological conditions, AR transactivation is
induced specifically by testosterone or DHT, causing AF2 in the AR
ligand binding domain to interact with the NH2-terminal LxxLL-like

sequence 2FQNLFL27 (15, 16, 21, 22). However, the AF2 hydrophobic
surface also forms an overlapping binding site for the LxxLL motifs of
the p160 coactivators. Under conditions of lower coactivator ex-
pression, AF2 would be occupied by the AR NH2-terminal domain
that could have the effect of inhibiting p160 coactivator recruitment
by AF2 (15). Ligand-induced interaction of AR AF2 with the p160
covators requires their overexpression, which results in increased
AR transactivation in transient transfection assays (16, 23). In recur-
rent prostate cancer growing in the absence of testis androgens,
increased coactivator expression could facilitate the induction of AR
transactivation by lower-affinity steroids such as the adrenal andro-
gen by shifting the equilibrium toward the formation of AR-TIF2
complexes. Increased AR transactivation induced by lower-affinity
ligands seems to result from coactivator-induced AR activity rather
than from a change in steroid binding affinity, inasmuch as previous
studies indicated that TIF2 overexpression does not alter the disso-
ciation rate of bound androgen.4 High expression of TIF2 and SRC1
in recurrent prostate cancer increases AR transactivation in response
to physiological concentrations of adrenal androgens or other steroids
with affinity for AR. Coactivation of AR transactivation may be
increased further by the phosphorylation of p160 coactivators (24,
25), thereby linking AR with growth factor signaling pathways. The
results provide a mechanism for AR-mediated tumor growth in recur-
rent prostate cancer.

Acknowledgments

We thank Natalie T. Bowen, John T. Minges, Qu Collins, Natalie Edmund,
and K. Michelle Cobb for technical assistance.

References

1. de Vere White, R., Meyers, F., Chi, S. G., Chamberlain, S., Siders, D., Lee, F.,
Stewart, S., and Guenrolock, P. H. Human androgen receptor expression in prostate
2. Ruizeveld de Winter, J. A., Trapman, J., Verme, M., Mulder, E., Zegers, N. D.,
and van der Kwaast, T. H. Androgen receptor expression in human tissues: an immuno-

3. Gregory, C. W., Hamil, K. G., Kim, D., Hall, S. H., Pretlow, T. G., Mohler, J. L.,
and French, F. S. Androgen receptor expression in androgen-independent prostate cancer
is associated with increased expression of androgen regulated genes. Cancer Res., 58:
Bartsch, G., and Klocker, H. Androgen receptor activation in prostate tumor cell lines
by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth
5. Nazareth, L. V., and Weigel, N. L. Activation of the human androgen receptor
through a protein kinase A signaling pathway. J. Biol. Chem., 271: 19900–19907,
1996.
6. Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. A mechanism for hormone-

independent prostate cancer through modulation of androgen receptor signaling by the
Mitogen-activated protein kinase kinase 1 activates androgen receptor-depen-
1999.
8. Sadar, M. D., Hussain, M., and Bruchovsky, N. Prostate cancer: molecular biology of
early progression to androgen independence. Endoc. Relat. Cancer, 6: 487–502,
1999.
Gunerlock, P. H., de Vere White, R. W., Pretlow, T. G., Harris, E. S., Wilson, E. M.,
Mohler, J. L., and French, F. S. Dehydroepiandrosterone activates mutant androgen
receptors expressed in the androgen-dependent human prostate cancer xenograft

4 B. He and E. M. Wilson, unpublished studies.


A Mechanism for Androgen Receptor-mediated Prostate Cancer Recurrence after Androgen Deprivation Therapy

Christopher W. Gregory, Bin He, Raymond T. Johnson, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/11/4315

Cited articles  This article cites 24 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/11/4315.full#ref-list-1

Citing articles  This article has been cited by 100 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/11/4315.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/61/11/4315.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.