Androgen-Dependent Regulation of Her-2/neu in Prostate Cancer Cells

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Abstract
The mechanisms underlying the progression of prostate cancer to a state of resistance to hormone ablation remain poorly understood. Here, we have investigated the relationship between androgen receptor (AR) and Her-2/neu in prostate cancer cells. Overexpression of Her-2/neu (c-ErbB2) activates the AR pathway and confers a survival and growth advantage to prostate cancer cells in an androgen-deficient milieu. In vitro, the absence of androgens or AR blockade induced Her-2/neu protein expression and phosphorylation. In contrast, upon readministration of androgens, Her-2/neu mRNA, protein, and phosphorylation levels decreased linearly with increasing concentrations of dihydrotestosterone as an advantage to prostate cancer cells. Overexpression of Her-2/neu (c-ErbB2) results in Her-2/neu overexpression. Similarly, knockdown of Her-2/neu led to induction of AR. However, when Her-2/neu and AR were simultaneously targeted, we observed cell death, whereas surviving cells retained low level expression of Her-2/neu. Thus, induction and activation of Her-2/neu occurs in an androgen-depleted environment or as a result of AR inactivation, promoting ablation-resistant survival of prostate cancer cells. These data provide the biochemical rationale to target Her-2/neu in hormone-refractory prostate cancer. (Cancer Res 2006; 66(11): 5723-8)

Introduction
Early-stage prostate cancers exhibit androgen dependence, and as a result of androgen withdrawal, prostate cancer cells undergo either cell cycle arrest or apoptosis. In the United States, prostate cancer patients with organ-confined disease are usually offered either radical prostatectomy or radiation therapy. In contrast, androgen ablation is the main form of therapy for men with metastatic prostate cancer. Although most prostate cancer patients respond at first to androgen ablation therapy (1), such patients eventually relapse and ultimately succumb to cancer (2). The mechanisms responsible for the initial survival and subsequent proliferation of ablation-resistant prostate cancer cells remain poorly characterized. Mutations of androgen receptor (AR) have been implicated in castration-resistant prostate cancer, but such mutations are rare and occur primarily under selective pressures of androgen deprivation (3).

Her-2/neu (also known as c-ErbB2) is a type 1 tyrosine kinase and a member of the ErbB receptor family, which also includes ErbB1/epidermal growth factor receptor, ErbB3, and ErbB4 (4). The 185-kDa Her-2/neu protein was originally identified as the transforming oncogene Neu (5). This form of Neu harbors a point mutation in the transmembrane domain that contributes directly to its oncogenic potential in rats (5). When overexpressed in the murine prostate, it causes the development of prostatic intraepithelial neoplasia that progresses to adenocarcinoma (6). Her-2/neu remains an orphan receptor, and its activation is thought to occur mainly through its hetero-oligomerization with other members of the ErbB receptor family. In fact, in the probasin-murine model, it heterodimerizes with ErbB3 (6). In the absence of androgens, Her-2/neu has been shown to confer growth advantage to prostate cancer cells (7). Alternatively, the oncogene Her-2/neu has been proposed as the survival factor for prostate cancer cells in an androgen-depleted environment (7–10). Furthermore, Her-2/neu has been linked to the activation of AR signaling and to the clinical progression of ablation-resistant human prostate cancer. Specifically, overexpression of Her-2/neu promotes the transactivation and phosphorylation of AR (6, 7, 11, 12), and stimulation of Her-2/neu stabilizes AR protein levels while enhancing the binding of AR to the promoters of androgen-regulated genes (13). Moreover, cells that overexpress Her-2/neu show evidence of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathway activation (12, 13), and in the presence of low androgen concentrations, PKI-166, a dual ErbB1/ErbB2 receptor tyrosine kinase inhibitor, showed profound growth-inhibitory effects on tumor growth, which could be partially reversed by the addition of androgen (13). Finally, the expression of Her-2/neu is higher in prostate cancers from patients who are treated with total androgen ablation therapy before undergoing surgery compared with untreated patients, whereas the highest level of expression is found in patients who developed disease that has become resistant to hormonal ablation (8–10). Therefore, Her-2/neu may play an important role in stimulating the AR pathway and in restoring AR function to prostate cancer cells in the absence of testosterone.

Although overexpression of Her-2/neu in breast and ovarian carcinomas involves the genomic amplification of the Her-2/neu...
locus (14), Her-2/neu overexpression in prostate cancer seems to result from its transcriptional activation (8). However, the mechanism of Her-2/neu induction following androgen withdrawal is not known. Here, we sought to determine whether Her-2/neu expression and activation are under direct androgen control in prostate cancer cells in vitro. We report that androgen signaling negatively regulates Her-2/neu in prostate cancer cells in vitro and in vivo, suggesting that Her-2/neu may promote prostate cancer cell survival following androgen ablation.

Materials and Methods

Cell culture. LNCaP prostate cancer cells [American Type Culture Collection (ATCC), Rockville, MD] were maintained in RPMI 1640 without phenol red and supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD). All experiments were done with 1% antibiotic-antimycotic. LNCaP cells used were from passages 25 to 45. Androgen depletion before dihydrotestosterone treatments was achieved by growing LNCaP cells in medium supplemented with 10% charcoal/dextran–treated FBS (cd-FBS; GeminiBioproducts, Calabasas, CA) for 48 hours. After 48 hours in a steroid-free environment, the cells were treated with 0, 0.1, 1, 10, 100 nmol/L and 1 µmol/L dihydrotestosterone (Sigma, St. Louis, MO) in ethanol for another 48 hours. The AR-negative prostate cancer line PC-3 and the AR-positive breast cancer cell line MCF-7 (ATCC) were used as controls. PC-3 cells were regularly maintained in F-12 medium with 8% FBS and MCF-7 cells in DMEM with 10% FBS. Before dihydrotestosterone treatments, their respective medium was replaced with RPMI 1640 with 10% cd-FBS and without phenol red for 48 hours. In addition, MCF-7 cells were also treated with 0, 100, and 1000 nmol/L 17β-estradiol (Sigma) in ethanol for 48 hours. The amount of ethanol added as a vehicle was 0.1% of the total volume. LNCaP cells were grown in RPMI 1640 supplemented with 10% FBS and 0.1 nmol/L dihydrotestosterone and subsequently treated with 10 µmol/L bicalutamide (Casodex, AstraZeneca, Wilmington, DE) for 0, 12, 24, and 48 hours.

Fluorescence-activated cell sorting analysis. Subconfluent cultures were pulse labeled for 1 hour with 10 µmol/L bromodeoxyuridine (BrdUrd; Boehringer Mannheim, Indianapolis, IN), harvested, washed twice with PBS, and fixed with 70% ethanol. Cells were washed twice with PBS and incubated with 2 mol/L HCl and 0.5% Triton X-100 for 30 minutes at 37°C. After washing with 0.1 mol/L sodium borate (borax), 2 × 106 cells were collected in PBS and stained with 100 µL fluorescein-conjugated anti-BrdUrd antibody (Boehringer Mannheim) for 30 minutes at room temperature. The cells were subsequently washed with PBS and stained with 1 µg/mL propidium iodide (Sigma) in the presence of RNase (DNase-free; Boehringer Mannheim). Cell cycle analysis was done on a fluorescence-activated cell sorter (FACScan, Becton Dickinson, San Jose, CA).

Protein extraction and Western blot analysis. Cells were scraped before protein extraction and Western blots were done as previously described (15). The following antibodies and their respective dilutions were used: anti-cyclin-dependent kinase 2 (cdk2; M2; 0.4 µg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cyclin D1 (CC12; 0.8 µg/mL; Calbiochem Oncogene, Cambridge, MA), anti-AR (PG-21; 1.0 µg/mL; Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-erbB2/Her-2 (1:1,000; Upstate Biotechnology), an anti-prostate-specific antigen (PSA: 1:500; Biogenex, San Ramon, CA), anti-β-actin (1:20,000; Sigma), retinoblastoma protein (Rb; 1:1,000; PharMingen, San Diego, CA). The anti-AR (1:2,000; DAKO, Inc., Carpinteria, CA) antibody used in this study is identical to the one from the Hercup test kit (DAKO) approved by the Food and Drug Administration for the evaluation of Her-2/neu expression in breast carcinoma.

Real-time quantitative reverse transcription–PCR (Taqman). RNA extraction and the reverse transcription were done as previously detailed (15). For each sample, a control reverse transcription–minus reaction was also done. After the reverse transcription step, the following specific primers and probe for the Her-2/neu cDNA were used for the real-time PCR: forward, 5′-AGGACCTCTGAACTGGTGTATG-3′; reverse, 5′-AGGCACAGTCCCTGT-3′; probe, 5′-VIC-AGATTGCCAAGGGGATGAGC-TCCTGG-TAMRA. Discrimination between cDNA and genomic DNA was enhanced by use of intron-spanning primers for Her-2/neu. In addition, the hybridization probe for Her-2/neu also spanned a splicing site to minimize annealing to genomic DNA. The housekeeping β-actin gene (Taqman human β-actin control reagents) was used as endogenous control to normalize the amount of Her-2/neu transcripts in each reaction. The PCR products were assayed for size and purity in a 2% agarose gel stained with ethidium bromide (data not shown). Real-time Taqman PCR was done using an ABI PRISM 7700 Sequence Detector. The Taqman PCR Core Reagent kit was used according to the manufacturer’s directions with the following modifications: dUTP was replaced by dTTP and incubation with AmpErase was omitted. PCR mixtures each contained 1 µL cDNA (equivalent to 50 ng of template RNA). 2.5 units AmpliTaq Gold, and 100 nmol/L (each) oligonucleotide primers and fluorogenic probe in a volume of 50 µL. After activation of AmpliTaq Gold for 10 minutes at 94°C, the amplification step consisted of 50 cycles of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 1 minute. All reagents were purchased from PE Applied Biosystems (Foster City, CA). In each experiment, five additional reactions with serial dilutions (×500 magnitude) of LNCaP cDNA as template were done with each set of primers and probes (Her-2/neu and β-actin) on the same 96-well plate to generate standard curves relating the threshold cycle (Ct) to the log input amount of template. All samples were run in triplicate. The relative amount of Her-2/neu transcripts in each sample was determined by using the standard curve method and by normalizing for β-actin mRNA expression levels, as previously described (8, 15).

Orthotopic implantation of tumor cells. Immunodeficient mice (Taconic, Germantown, NY) were anesthetized with Avertin (Sigma), a low-midline incision was made, and LNCaP (5 × 106/10 µL) in RPMI 1640 were implanted into the ventral prostate lobes using a 30-gauge needle with a 0.1-µL syringe. The prostate was returned to the abdominal cavity, and the abdominal wall was sutured closed. Testosterone pellet (Innovative Research of America, Sarasota, FL) were s.c. implanted on the same day. Surgical castration was done 6 weeks after orthotopic implantations, and the testosterone pellets were removed.

Surgical castration. An incision was made in the scrotum of anesthetized mice and in the tunica. The testis, vas deferens, and attached testicular fat pad were pulled out of the incision, and the blood vessels supplying the testis were cauterized. The testis, vas deferens, and fatty tissue were severed just below the site of the cauterization, and this procedure was repeated on the contralateral side before suturing the scrotum. Mice were sacrificed at 0, 1, 2, 3, and 4 weeks after the surgical castration, and a blood sample was taken from each mouse. Testosterone and PSA levels were measured by ELISA (ALPCO Diagnostic, Windham, NH). All animal work was done in accordance to our institutional animal care committee guidelines.

RNA interference. A lentiviral vector (pLKO.puro) developed by the RNA interference Consortium (Broad Institute of Massachusetts Institute of Technology and Harvard) was used to introduce specific short hairpin RNAs (shRNA) into LNCaP cells. The oligonucleotide sequences used to target specific genes are AR, 5′-CGGACTACATACTTCTTCA-3′; HER-2/neu, 5′-TGTCAGATCCAGCTTGTGA-3′.

Lentiviruses were generated using the packaging cell line 293T FT, the pLKO.puro derivatives, and two packaging plasmids (pCMVΔA8.91 and pMD.G/VSVG). Supernatants containing viral particles were collected at 36 and 48 hours and pooled. Viral supernatants were diluted 1:6 and supplemented with polybrene (5 µg/mL final concentration). Target cells grown in RPMI were serologically infected twice and selected with 1.5 µg/mL puromycin (final concentration) for 3 days. The first round included hairpins against either HER-2/neu or control green fluorescent protein (GFP). After puromycin selection, each one of these two cell types was further infected with hairpins against either AR or control GFP. The four populations obtained were shGFP/shGFP, shHER-2/shGFP, shGFP/shAR, and shHER-2/shAR.

Results and Discussion

We previously showed that the progression of prostate cancer to castration resistance correlates with a gradual increase in Her-2/neu expression in tumor cells (8). To investigate whether AR
signaling directly regulates Her-2/neu expression and activation, we chose the LNCaP cell line as a model for prostate cancer. LNCaP cells harbor an AR allele with a point mutation, which renders the AR protein sensitive to activation by estrogen, progesterone, and flutamide in addition to androgens (16, 17). After growing LNCaP cells in cd-FBS for 48 hours to deplete the medium of any steroid hormones, dihydrotestosterone was added back at various concentrations for 48 hours. Peak proliferation occurred at low doses of dihydrotestosterone (0.1, 1, and 10 nmol/L), whereas high doses of dihydrotestosterone (100 and 1,000 nmol/L) induced G1 arrest of LNCaP cells (Fig. 1A and B), consistent with previous observations (16). Peak proliferation correlated with the hyperphosphorylation of the RB and the induction of cell cycle proteins: cyclin D1 and Cdk2 (Fig. 1B). These observations recapitulate other studies, where low doses of dihydrotestosterone increased the expression of Cdk2 and Cdk4 genes and induced proliferation of LNCaP cells (18).

In contrast to proliferating cells, we observed hypophosphorylation of RB and down-regulation of Cdk2 and cyclin D1 in G1-arrested cells that were treated with high levels of dihydrotestosterone. Concurrent with G1 arrest, LNCaP cells exhibited a more differentiated phenotype, as the expression of PSA, which reflects AR activation and prostate luminal cell differentiation, increased linearly with the exposure to higher concentrations of dihydrotestosterone (Fig. 1B). In contrast, we found higher levels of both total Her-2/neu expression and phospho-Her-2/neu levels in cells grown in cd-FBS (Fig. 1B, lane 2) compared with those grown in regular FBS (Fig. 1B, lane 1). Upon readdition of dihydrotestosterone, Her-2/neu levels decreased linearly with increasing concentrations of dihydrotestosterone (Fig. 1B, lanes 3–7). As expected, we found no changes in the expression of the β-actin. Thus, Her-2/neu is up-regulated in the absence of androgens, whereas dihydrotestosterone down-regulates Her-2/neu expression.

To examine the mechanisms of Her-2/neu regulation in response to androgens, Her-2/neu mRNA levels were measured by real-time quantitative reverse transcription-PCR (RT-PCR) with β-actin as a reference gene. Androgen depletion induced Her-2/neu mRNA levels in LNCaP cells, and treating the cells with increasing concentrations of dihydrotestosterone for 48 hours down-regulated Her-2/neu mRNA levels (Fig. 1C). These results correlated with Her-2/neu protein levels (compare Fig. 1B and C), suggesting that androgen stimulation regulates Her-2/neu expression at least in part by altering Her-2/neu transcript levels.

To examine whether the presence of functional AR protein was necessary for androgens to induce down-regulation of Her-2/neu expression levels, we initially starved cells of androgens to activate Her-2/neu in LNCaP (Fig. 2A, lane 2). As expected, Her-2/neu and phospho-Her-2/neu were increased after incubation for 48 hours in charcoal-stripped serum (Fig. 2A, lane 2). We also investigated the effects of treating LNCaP cells that had been grown in charcoal-stripped serum with 1,000 nmol/L dihydrotestosterone over 48 hours (6, 12, 24, and 48 hours). This concentration of dihydrotestosterone induced G1 arrest and hypophosphorylation of RB (Fig. 2A). Her-2/neu protein levels gradually decreased with time and were lowest after exposure to 1,000 nmol/L dihydrotestosterone for 48 hours. In contrast, we found that with this high, “differentiating” dose of dihydrotestosterone, PSA protein levels increased, peaking at 48 hours. As expected, this pattern of PSA expression inversely correlated with Her-2/neu levels (Fig. 2A). In keeping with the finding that Her-2/neu is transcriptionally regulated (Fig. 1), down-regulation of Her-2/neu mRNA preceded protein down-regulation (Fig. 2B), as peak Her-2/neu mRNA and protein down-regulation occurred after 24 and 48 hours of androgen treatment, respectively. To confirm that functional AR
was necessary for the down-regulation of Her-2/neu, we then treated AR expressing MCF-7 cells and the AR-negative PC-3 cells with androgens. Exposing MCF-7 cells to 100 and 1,000 nmol/L of dihydrotestosterone down-regulated Her-2/neu levels and induced AR expression (Fig. 2, lanes 2 and 3). Similarly, we observed down-regulation of Her-2/neu in estrogen-treated MCF-7 cells (Fig. 2C, lanes 4 and 5). However, after treating PC-3 cells with increasing concentrations of dihydrotestosterone, no changes were seen in the levels of Her-2/neu (data not shown). Thus, these results further corroborate that the conclusion that Her-2/neu regulation is mediated by androgens. In addition, these findings confirm that regulation is at least in part transcriptional, although an effect on mRNA stability cannot be excluded. Finally and importantly, these results indicate that the presence of a functional AR protein is necessary for androgens to down-regulate Her-2/neu expression levels.

AR expression (Fig. 2C, lanes 2 and 3). Similarly, we observed down-regulation of Her-2/neu in estrogen-treated MCF-7 cells (Fig. 2C, lanes 4 and 5). However, after treating PC-3 cells with increasing concentrations of dihydrotestosterone, no changes were seen in the levels of Her-2/neu (data not shown). Thus, these results further corroborate that the conclusion that Her-2/neu regulation is mediated by androgens. In addition, these findings confirm that regulation is at least in part transcriptional, although an effect on mRNA stability cannot be excluded. Finally and importantly, these results indicate that the presence of a functional AR protein is necessary for androgens to down-regulate Her-2/neu expression levels.

To test whether the prostatic microenvironment influenced Her-2/neu regulation by AR, we placed LNCaP cells orthotopically
in the ventral prostate of immunodeficient mice in the presence and absence of supplemental testosterone. In this system, tumors are visible 6 weeks after injection (19). After allowing such tumors to grow for 6 weeks, we castrated a subset of the mice harboring such tumors and measured Her-2/neu levels at various times after castration. Her-2/neu expression in the orthotopic tumors was significantly up-regulated 2 to 4 weeks after castration over the control mice. This increase in Her-2/neu levels was accompanied by a corresponding increase in serum PSA levels and was inversely correlated to serum testosterone (Fig. 3). These results provide in vivo evidence of the biological effects of the Her-2/neu pathway on AR stabilization and induction of its transcriptional activity. These results are in keeping with other studies that have shown up-regulation of PSA and activation of the AR pathway upon overexpression of Her-2/neu in LNCaP cells (7).

To verify that Her-2/neu is regulated by the AR signaling pathway, we studied the effect of the anti-androgen drug bicalutamide (Casodex) on the proliferation as well as on Her-2/neu mRNA and protein expression of LNCaP cells. Bicalutamide has been used as a means of androgen ablation in the treatment of prostate cancer patients by blocking the androgen-mediated activation of AR (20). Treatment of LNCaP cells with 10 μmol/L Casodex for 0, 12, 24, and 48 hours reduced proliferation significantly as measured by the percentage of cells in G0-G1, S, and G2-M phases of the cell cycle (Fig. 4). Concurrent with a decrease in proliferation, treatment with bicalutamide decreased the phosphorylated form of Rb and blocked PSA and AR protein expression (Fig. 4A). In contrast, bicalutamide exposure induced Her-2/neu protein levels, which peaked 48 hours after treatment (Fig. 4A). Furthermore, consistent with an increase in Her-2/neu protein levels, treatment of LNCaP with 10 μmol/L bicalutamide progressively increased both Her-2/neu phosphorylation and mRNA levels as measured by real-time quantitative RT-PCR (Fig. 4B). Taken together, these results further substantiate the fact that negative regulation of Her-2/neu by androgens occurs at both the transcriptional and posttranslational levels via the AR signaling pathway.

We next tested whether direct down-regulation of AR would result in Her-2/neu up-regulation. To accomplish this, we suppressed the expression of AR using shRNA specific for AR and selecting cells that stably express these shRNA in both RPMI
(Fig. 5A) and charcoal-stripped serum (not shown). Consistent with the above data, we found that down-regulation of AR resulted in increased expression of Her-2/neu in both RPMI and charcoal-stripped serum. Just as suppression of AR led to increased Her-2/neu expression knockdown of Her-2/neu led to induction of AR. However, when both AR and Her-2/neu hairpins were used in combination, Her-2/neu seemed to be required by cells to survive. Specifically, we initially observed increased cell death after the simultaneous exposure to the shRNAs targeting AR and Her-2/neu. Thereafter, the cells expressing Her-2/neu were selected for and survived. We were unable to obtain cells that lacked expression of both AR and Her-2/neu when both genes were targeted. We also measured the proliferation of cells lacking AR and Her-2/neu in response to synthetic androgens over 9 days (Fig. 5B). Down-regulation of both AR and Her-2/neu (or of either alone) resulted in complete lack of androgen-induced cell proliferation. These data suggest when both AR and Her-2 are targeted cell survival is impaired. Furthermore, these experiments provide evidence that in the presence of a wild type and functional AR, as is presumably the case in the majority of ablation-resistant human prostate cancers, targeting of Her-2/neu affects the proliferative response of prostate cancer cells to any circulating androgens.

Tyrosine phosphorylation regulates the activity of many receptor tyrosine kinases. Specific phosphorylation sites serve as binding sites for Src-homology (SH2) domains located in downstream signaling molecular. The recruitment of these molecules to an activated membrane receptor or associated docking proteins occurs when the SH2 domains of downstream molecules specifically bind to phosphotyrosine residues of either the membrane receptor or an upstream molecule. Her-2/neu is phosphorylated and activated by its heterodimerization with other members of the erbB receptor family (21). We found that the total levels of Her-2/neu correlated with the levels of its active, phosphorylated form. Phosphorylation of Her-2/neu, in turn, activates signaling pathways, such as the MAPK and PI3K/Akt pathways, which confer survival to prostate cancer cells (11, 12). In addition, Her-2/neu stabilizes AR protein levels and optimizes binding of AR to promoter/enhancer regions of androgen-regulated genes (13). In keeping with these findings, we found that serum PSA levels correlating with Her-2/neu overexpression occur 2 to 4 weeks after castration in a mouse model in which LNCaP cells are injected orthotopically.

These observations suggest that induction and activation of Her-2/neu occurs as a result of AR inactivation. Although androgen withdrawal promotes G₁ arrest and/or apoptosis of many androgen-dependent prostate cancers, a subset of cells survive and may overexpress and activate Her-2/neu following androgen ablation, as it is the case in vivo (8–10). Here, we provide the molecular mechanisms for the androgen regulation of Her-2/neu within prostate cancer cells. Interfering with the crosstalk between the transactivation of AR and the Her-2/neu signaling pathway may thus represent a valid therapeutic option for prostate cancer.

Acknowledgments

Received 11/1/2005; revised 2/22/2006; accepted 3/9/2006.

Grant support: NIH Specialized Programs of Research Excellence grants SPOCA08381 and PO1 CA089021, Department of Defense grant W81XWH-06-1-0053, and the Prostate Cancer Foundation (M. Loda, W.C. Hahn, and S. Signoretti).

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