Attenuation of Ras Signaling Restores Androgen Sensitivity to Hormone-refractory C4-2 Prostate Cancer Cells

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INTRODUCTION

Progression of prostate cancer to androgen-refractory disease is correlated with increased expression of growth factors and receptors capable of establishing autocrine and/or paracrine growth-stimulatory loops. Many of these growth factor receptors engage Ras as part of their normal signaling activities, raising the possibility that activation of endogenous c-Ras could be a common mechanism for prostate cancer progression. Here we demonstrate that inducible expression of a dominant negative form of Ras restores androgen sensitivity to a hormone-refractory prostate cancer cell line. We show that expression of RasN17 in the hormone-refractory C4-2 cell line enhances in vitro sensitivity to the growth-inhibitory action of the antiandrogen Casodex and inhibits anchorage-independent cell growth. Moreover, although induction of RasN17 by itself has no observable effect on the growth of C4-2 xenografts in intact male mice, it restores androgen dependence to the C4-2 xenografts so that they dramatically regress after surgical androgen ablation.

ABSTRACT

Progression of prostate cancer to androgen-refractory disease is correlated with increased expression of growth factors and receptors capable of establishing autocrine and/or paracrine growth-stimulatory loops. Many of these growth factor receptors engage Ras as part of their normal signaling activities, raising the possibility that activation of endogenous c-Ras could be a common mechanism for prostate cancer progression. Here we demonstrate that inducible expression of a dominant negative form of Ras restores androgen sensitivity to a hormone-refractory prostate cancer cell line. We show that expression of RasN17 in the hormone-refractory C4-2 cell line enhances in vitro sensitivity to the growth-inhibitory action of the antiandrogen Casodex and inhibits anchorage-independent cell growth. Moreover, although induction of RasN17 by itself has no observable effect on the growth of C4-2 xenografts in intact male mice, it restores androgen dependence to the C4-2 xenografts so that they dramatically regress after surgical androgen ablation.

MATERIALS AND METHODS

Establishment of pTetOn RasN17 C4-2 Cell Line. The androgen-independent LNCaP cell line derivative, C4-2, was obtained as a generous gift from L. Chung (Emory University). The DOX-inducible C4-2 primary cell line (C4-2 pTetOn) was created according to the manufacturer’s instructions (pTetOn; Clontech) using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate (Boehringer Mannheim) transfection reagent. Thirty G418-resistant clones were isolated and further screened for DOX inducibility via transient transfection with the tetracycline-responsive pTRE-Luc reporter vector. Cell luciferase activity was screened for high inducibility and low background. In this manner, one clone was isolated with >300-fold inducible luciferase activity and undetectable background activity. This clone was used for establishment of the double-stable inducible C4-2 pTetOn RasN17 cell line.

DOUBLE-STABLE pTETON RASN17 CELLS WERE CREATED BY DOUBLE TRANSFECTION OF pTRE-RASN17 AND THE PTK-HYg HYGROMYCIN RESISTANCE VECTOR IN A 20:1 RATIO (1 µg total DNA/200,000 cells) ACCORDING TO THE MANUFACTURER’S PROTOCOL. AFTER 72 H IN 10% FBS RPMI 1640, HYGROMYCIN WAS ADDED AT A FINAL CONCENTRATION OF 100 UNITS/mL (100 µg/mL) IN 10% FBS RPMI 1640. OF 41 INDIVIDUAL DOUBLE-STABLE CLONES, 1 CLONE REVEALED HIGH-LEVEL INDUCIBLE RASN17 EXPRESSION WITH NO DETECTABLE BACKGROUND. THIS CELL LINE WAS EXPANDED AND USED FOR EXPERIMENTAL ASSAYS. CELLS WERE MAINTAINED AT 37°C AND 5% CO2.

MTT Cell Proliferation Assays. Cells were grown in RPMI 1640, C4-2 control or pTetOn RasN17 cells in 10% FBS were plated at a concentration of 15,000 cells/well on 24-well tissue culture plates (Primaria; Becton Dickinson). Cells were allowed to adhere for 24 h in 10% FBS, with or without 1 µg/ml DOX. Media were changed to 2% FBS with or without 30 µM Casodex, alone or in combination with continued 1 µg/ml DOX administration. No further manipulation of cell culture conditions occurred. Relative cell numbers were quantified every 2 days via MTT analysis as described previously (8).

Agarose Assay. C4-2 pTetOn RasN17 cells growing for 24 h in 10% FBS with or without 1 µg/ml DOX were resuspended in 1 ml of 0.5% agarose (SeaPlaque; FMC BioProducts) supplemented with 2% normal FBS (Life Technologies, Inc.), and layered on top of

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4 The abbreviations used are: MAPK, mitogen-activated protein kinase; PSA, prostate-specific antigen; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AR, androgen receptor; DOX, doxycycline; nFBS, normal fetal bovine serum; ARE, androgen response element.
a pre-gelled bottom layer of identical composition according to established methods (8). Final cell number was approximately 25,000 cells/35-mm tissue culture dish. Thirty μl of the antiandrogen Casodex, 20 μg of the mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor U0126, or equivalent control vehicle, DMSO, was included in agarose gel at the time of plating. The DMSO concentration was kept at a constant 0.1%. Colonies were quantified after 3 weeks of incubation at 37°C and 5% CO2 according to previously established methods.

**Northern Analysis.** Total RNA was isolated from subconfluent cultures of RasN17 cells after 4 days of culture using Qiagen RNeasy. Subsequent Northern analysis was performed according to established protocols (8).

**Animal Studies and Immunohistochemistry.** Male 5–6-week-old BALB/cAnGr-lucBR mice (National Cancer Institute) were bilaterally inoculated s.c. with 2 × 106 pTetOn RasN17 cells in Matrigel (50:50 volume) for a total volume of 100 μl injection site. Palpable tumors were observed as early as 2 weeks after injection. Animals were included in experimental assays (see below) when tumor burden was, on average, ≥7 mm in diameter.

Tumor-bearing animals were divided into two groups. Group 1 received DOX in drinking water every 5 days (final concentration, 100 μg/ml) in addition to s.c. implantation of a 60-day time release DOX tablet (42 mg/pellet; Innovative Research of America, Sarasota, FL). Group 2 received sterile water control vehicle in addition to sham pellet implantation. The above-mentioned groups were further segregated and either surgically castrated or sham castrated 1 day after DOX administration. Bilateral castration was performed on ketamine/xylazine/acepromazine-anesthetized mice using standard surgical techniques. Tumor burden was then recorded weekly in two dimensions and recorded as average diameter. Upon completion of the experiment, mice were sacrificed using carbon dioxide asphyxiation and placed on ice, and the tumors were excised and bisected. Tumors were fixed in Zn2+-buffered formalin for 48 h, followed by paraffin embedding. In vivo expression of epitope-tagged RasN17 was observed using peroxidase preconjugated M2 anti-FLAG antibody (Sigma) at a 1:200 dilution.

Alternatively, tumor samples for Western analysis were snap frozen in liquid nitrogen, ground with mortar and pestle, resuspended in 0.5% SDS-PAGE lysis buffer, and then sonicated on ice with eight 1-s bursts. Samples were subsequently microcentrifuged at 4°C for 1 min, and lysate was removed from residual precipitated tissue and subjected to standard SDS-PAGE on a 10% polyacrylamide gel. Serum PSA levels were quantified using an automated Abbott Laboratories IMX MEIA clinical assay machine.

**Antibodies and Immunofluorescence.** These were as described previously (8).

**Statistical Analysis.** Profiles of tumor volume were modeled using a random coefficient regression model that incorporates all available posttreatment data for each animal (12). The curves were estimated as restricted cubic spline functions of time since treatment (in weeks) with 4 knots, which required 3 degrees of freedom/treatment group. The random coefficient regression model, in essence, estimates and averages curves calculated for each animal within each treatment group, accounting for the correlation among the repeated measurements over time. The model also included a term for pretreatment tumor volume (calculated at week 4), which served as a baseline adjustment. The model was fit using the MIXED procedure in SAS Version 8.2 (SAS Institute, Cary, NC).

Statistical analyses of agorose experiments were carried out using standard ANOVA. Type 3 F-tests were used to test for differences among curves overall and within each treatment group. Fisher’s least significant difference method was used to generate pairwise comparisons between treatment groups at each time point posttreatment. The model was fit using the MIXED procedure in SAS Version 8.2 (SAS Institute, Cary, NC).

**RESULTS**

**Characterization of the C4-2 pTetOn RasN17 Cell Line.** Because stable expression of dominant negative RasN17 might prove generally cytostatic or cytotoxic, we chose to engineer a derivative of the prostate cancer C4-2 cell line in which RasN17 was under the inducible control of DOX (see “Materials and Methods”). In the absence of DOX, all growth parameters and AR expression levels of C4-2 pTetOn RasN17 cells were identical to parental C4-2 as well as the single-stable C4-2 pTetOn cell line (data not shown). Western blotting revealed significant RasN17 expression after 24-h exposure to as little as 0.25 μg/ml DOX (Fig. 1A), with maximal 24 h expression occurring after exposure to 1 μg/ml DOX (Fig. 1B). The addition of higher levels of DOX did not significantly increase RasN17 expression levels (data not shown). Substantial RasN17 expression was detected by 12 h after addition of 1 μg/ml DOX to the culture medium (Fig. 1B). Administration of a one-time 1 μg/ml dose of DOX to cells in culture resulted in high-level RasN17 expression maintained after 7 days in culture (data not shown), demonstrating that Ras induction is functionally stable in serum-supplemented culture media over long time periods. This could be due to the stability of DOX, RasN17, or a combination of the two. RasN17 protein levels were maintained for at least 1 day after DOX removal (data not shown).

Western analysis with a pan-Ras antibody revealed RasN17 expression at approximately one-third the level of endogenous c-Ras (Fig. 1C). The ability of RasN17 to induce phenotypic alterations at substoichiometric levels has been described previously (13). Presumably this is because RasN17 inhibits signaling by forming inactive complexes with upstream catalytic activators (14).

Indirect in vitro immunofluorescence revealed uniform membrane and cytosolic staining of virtually 100% of cells upon induction with 1 μg/ml DOX (data not shown). Although Ras is generally thought of as a membrane protein, cytosolic fractions have also been observed, including GDP-bound Ras (15). Importantly, RasN17 expression was able to attenuate endogenous MAPK phosphorylation resulting from 5-min exposure to EGF (Fig. 2), demonstrating that the dominant negative effect is operative in these cells.

**Expression of RasN17 Complements Growth-inhibitory Properties of Antiandrogen Administration.** We asked whether attenuation of endogenous Ras activity would sensitize hormone-refrac-

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**Fig. 1.** Characterization of the C4-2 pTetOn Ras N17 cell line. A, C4-2 pTetOn RasN17 cells were treated with various concentrations of DOX for 24 h. Cells were solubilized, and RasN17 expression was characterized by epitope SDSL-PAGE Western analysis using anti-FLAG M2 monoclonal antibody. B, C4-2 pTetOn Ras N17 time course expression of FLAG-tagged RasN17. Cells were induced to express RasN17 by the addition of 1 μg/ml DOX. Time-dependent expression of RasN17 was detected by anti-FLAG Western blotting of whole cell lysates. C, relative expression levels of RasN17 to endogenous c-Ras. C4-2 pTetOn RasN17 cells were grown for 24 h in 1 μg/ml DOX-supplemented media and then subjected to Western analysis with a pan-Ras antibody (Upstate Biotechnology). The mutant Ras migrates at a lower mobility (top band, +Dox).

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tory C4-2 prostate cancer cells to the antiproliferative effects of the antiandrogen Casodex. C4-2 cells are sensitive to pharmacological inhibition of HER-2/neu (16), implying that autocrine-driven progression via a Ras-dependent signaling mechanism might contribute to the observed hormone-refractory phenotype.

Control experiments revealed that DOX treatment did not cause significant in vitro growth inhibition of either C4-2 parental cells (Fig. 3A) or single-stable C4-2 pTetOn RasN17 cells (data not shown). However, administration of DOX alone to the inducible pTetOn RasN17 cells resulted in a partial dose-dependent inhibition of growth (Fig. 3B). Both parental C4-2 and C4-2 pTetOn RasN17 cells demonstrated a 30% reduction in cell proliferation in response to 30 μM Casodex, reflecting the residual androgen responsiveness of these cells. However, induction of RasN17 (1 μg/ml DOX) in combination with 30 μM Casodex resulted in near complete inhibition of C4-2 pTetOn RasN17 in vitro growth. Expression of RasN17 was confirmed via Western analysis of cell lysates after growth assays were completed (data not shown).

We conclude that attenuation of Ras activation by expression of RasN17 is sufficient to enhance the androgen sensitivity of C4-2 prostate cancer epithelial cells, making them more susceptible to the antiproliferative effects of the antiandrogen Casodex.

Expression of RasN17 Complements Ability of Antiandrogen Administration to Inhibit Anchorage-independent Growth. Anchorage-independent growth is a hallmark of malignant transformation. Cell anchorage allows efficient signal propagation between Ras and downstream effector kinases such as MAPK (17). We asked whether expression of RasN17 was sufficient to enhance the androgen dependence of C4-2 cells under nonadherent conditions.

The addition of DOX to 5% nFBS-supplemented agarose cultures inhibited soft agarose colony formation in pTetOn RasN17 cells but not in parental C4-2 cells (Fig. 4A). In accordance with their residual androgen-responsive phenotype, administration of 30
μM Casodex alone resulted in a modest reduction in colony number of both C4-2 parental and RasN17 cells (Fig. 4B). Whereas addition of a submaximal amount of DOX (0.25 μg/ml) promoted a nearly 50% reduction in agarose colonies in pTetOnRasN17 cells, combined addition of 0.25 μg/ml DOX and 30 μM Casodex completely inhibited colony formation of pTetOn RasN17 cells, but not parental C4-2 cells. All experiments were performed in parallel with the pTetOn C4-2 single-stable cell line as a control. We observed no significant difference in growth rates between parental C4-2 and the C4-2 pTetOn single-stable cell line for this and all subsequent experiments.

We conclude that attenuation of Ras activation by expression of dominant negative RasN17 is sufficient to block anchorage-independent growth and can sensitize C4-2 cells to the antiproliferative effects of antiandrogens under nonadherent conditions.

**Expression of RasN17 Does Not Attenuate Endogenous Cellular PSA Expression and Transcription.** PSA is the product of an androgen-responsive gene and is used as a marker for prostate cancer diagnosis and for recurrence of hormone-refractory disease. Because PSA production is used as a surrogate for prostate cancer growth, we hypothesized that expression of RasN17 would have effects on PSA expression comparable with the effects on growth described above: attenuation of Ras signaling alone was expected to reduce PSA expression and, when combined with antiandrogen, might further reduce endogenous PSA to levels below that achieved with either agent alone.

Subconfluent cultures of C4-2 pTetOn RasN17 cells were grown for 4 days under serum-free conditions in the presence or absence of 1 μg/ml DOX with or without concomitant 30 μM Casodex administration. Whereas serum starvation resulted in a significant reduction in basal PSA protein and mRNA levels, maximal induction of RasN17 alone did not further reduce PSA protein expression under serum-free or serum-supplemented conditions (data not shown). Curiously, 4-day Casodex administration, alone or in combination with DOX, also failed to reduce endogenous cellular PSA levels under both serum-starved and serum-supplemented culture conditions (data not shown). Equal protein loading was confirmed by blotting with a MAPK-specific monoclonal antibody. Northern analysis confirmed that PSA mRNA levels were similarly unaffected by RasN17 expression, Casodex administration, or a combination of the two under serum-starved or serum-supplemented conditions (data not shown).

**Expression of RasN17 Complements Surgical Androgen Ablation in Causing Tumor Regression.** Tumors formed by C4-2 cells are refractory to hormone ablation monotherapy produced by castration. We asked whether in vivo expression of RasN17 could restore androgen dependence to these xenografts so that they would regress after castration, as do tumors formed from the parental androgen-dependent LNCaP cells (18).

Nude male mice received bilateral injection with either C4-2 or pTetOn-RasN17 cells in Matrigel. After tumors reached an average diameter of 7 mm, mice were castrated or sham castrated. Both castrated and sham-castrated mice were further segregated, and half were given DOX by implantation of time release DOX pellets in addition to having their drinking water supplemented with DOX. Sham pellet implantation was used as a control. Highlighting their hormone-refractory nature, parental C4-2 (data not shown) and pTetOn RasN17 tumors grew in an unrestricted manner in both intact and castrated control mice (Fig. 5A). Similarly, intact mice given DOX alone did not demonstrate any tumor regression in either parental C4-2 or pTetOn RasN17 cells.

Castrated mice treated with DOX displayed complete tumor regression in 80% of tumors (and stasis in the other 20%) within 3–4 weeks posttreatment. No tumor rebound was observed once DOX was removed from the drinking water for over 2 months. Anti-epitope immunohistochemistry of formalin-fixed tumor samples revealed RasN17 expression in a subpopulation of tumor cells from animals treated with DOX (Fig. 5B).

Statistical analysis revealed significant differences in posttreatment tumor volume curves among the treatments after adjusting for baseline tumor volume (P < 0.001). Further investigation showed that this difference was driven by the DOX + Casodex group and that the curve for the DOX + Casodex group differed significantly from all other treatment groups (P < 0.001). No other treatment group curves differed significantly from one another. The differences between DOX + Casodex and the other treatments were obvious soon after treatment and significant at the P < 0.001 level from week 2.5 onward.

In sum, we demonstrate that suppression of Ras activation can hypersensitize C4-2 tumors to the antiproliferative effects of androgen ablation therapy, resulting in either tumor stasis or significant regression of C4-2 tumors in nude mice.

**DISCUSSION**

**Expression of RasN17 Sensitizes C4-2 Cell Growth to the Antiandrogen Casodex.** Functional ARs are commonly maintained throughout the transition to androgen-refractory prostate cancer and are frequently overexpressed and mutated to respond to a broader array of agonists (19–33). These observations strongly suggest that the AR maintains a continuing role even in “androgen-independent” cancers. This is in agreement with recent reports showing that the AR is critical for growth of androgen-refractory cells (34, 35) and with our finding that Ras expression renders LNCaP cells hypersensitive to low levels of androgen rather than fully androgen independent (8). In this study, we examine the role of Ras in the androgen responsiveness of C4-2 prostate epithelial cells. C4-2 cells express a functional AR and nonmutationally active c-Ras yet display hormone-refractory growth (11).

We found that DOX-mediated induction of RasN17 had a dose-dependent inhibitory effect on growth of C4-2 pTetOn RasN17 cells. Administration of Casodex alone similarly resulted in moderate growth inhibition, confirming the continuing functional role of the AR in these cells. Expression of RasN17 in combination with Casodex resulted in nearly complete inhibition of both anchorage-dependent and -independent growth of pTetOn RasN17 cells. We suggest a mechanism where RasN17 expression attenuates growth factor signaling cascades to such an extent that the residual androgen-sensitive nature of C4-2 cells is revealed, thus restoring the antiproliferative properties of Casodex.

**RasN17 Expression Fails to Inhibit Endogenous Cellular PSA Expression.** PSA is an androgen-regulated serine protease whose elevated expression is correlated with androgen-independent growth after hormone ablation therapy failure. Enhanced growth factor receptor activity is associated with androgen-independent PSA gene expression (36), and agonists that can activate the Ras to MAPK pathway have been shown to enhance reporter activity of the AR on the PSA promoter and AREs from the PSA promoter (37, 38). We have also found that expression of mutationally activated Ras in LNCaP cells renders PSA production hypersensitive to low levels of androgen (8). We therefore expected that attenuation of Ras signaling via expression of RasN17 would attenuate PSA expression as it attenuated growth.

To our surprise, whereas removal of serum resulted in a significant reduction in PSA production, expression of RasN17 in serum-free or serum-containing media did not measurably affect PSA mRNA tran-
scription or protein expression. Because serum components likely regulate transcription of a number of gene promoters, including PSA, we propose that C4-2 PSA expression occurs in part via serum-regulated, Ras-independent mechanisms. Indeed, nuclear factor κB has been proposed as an alternative mechanism for up-regulating PSA promoter activity in androgen-independent xenografts (39). We further demonstrate that 4-day 30 μM Casodex administration did not additionally inhibit PSA expression in either serum-free or serum-supplemented culture conditions. From this, we conclude that PSA expression in C4-2 cells may be in part independent of AR activity and may involve AR-associated cofactors or other promoter-regulatory regions. Indeed, a 150-bp region of the C4-2 PSA promoter was demonstrated as a major regulatory element independent of AR activity (40).

Androgen-regulated cellular growth has previously been reported to be separable from androgen-regulated expression of at least some genes, including PSA (41–43). The fact that activation of Ras signaling can turn on PSA gene expression in LNCaP cells (8) but inhibition of Ras signaling with RasN17 fails to attenuate PSA expression in the C4-2 cells, even though the C4-2 cells were derived from LNCaP,
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presumably reflects the fact that Ras activation was not selected for in derivation of C4-2. Thus, there may be additional regulatory alterations that have occurred in these cells. Nevertheless, androgen dependence of growth and tumorigenicity are sensitive to RasN17 expression, indicating that Ras function is still necessary for growth regulation in these cells. In addition, the work presented here reinforces a clinically important distinction between PSA expression and cellular proliferation.

Inducible Expression of RasN17 Complements Surgical Androgen Ablation in Mediating Tumor Regression. The most striking effect of RasN17 expression was seen in xenografts. Here, RasN17 expression or surgical castration alone was without measurable effect on tumor growth, whereas simultaneous castration and induction of RasN17 caused almost complete and irreversible tumor regression. Taken together with our in vitro results, these findings indicate that Ras N17 expression restores androgen dependence to a previously characterized hormone-refractory prostate cancer cell line. The clinical implication of these findings is that inhibition of Ras signaling might be a very useful therapeutic adjunct to androgen ablation in the treatment of hormone-refractory prostate cancer. We have found that effector loop mutants of Ras that selectively activate the MAPK pathway render LNCaP cells hypersensitive to androgen (8). This suggests that the MAPK arm of Ras signaling may be the appropriate target for advanced prostate cancer. We are currently evaluating this prediction.

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