NE-10 Neuroendocrine Cancer Promotes the LNCaP Xenograft Growth in Castrated Mice

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INTRODUCTION
Prostate cancer growth is initially promoted by androgens. Withdrawal of androgens through physical or chemical castration usually leads to regression of the disease. This regression is transient, because the cancer becomes androgen refractory and progresses to androgen-independent growth. It is still unclear why prostate tumors eventually become androgen refractory.

Neuroendocrine (NE) cells are present in the normal and neoplastic prostate (1). Increases in NE phenotype and secretory products are closely correlated with tumor progression and androgen-independent prostate cancer. However, the mechanisms by which NE cells influence prostate cancer growth and progression, especially after androgen ablation therapy, are poorly understood. To investigate the role of NE cells on prostate cancer growth, LNCaP xenograft tumors were implanted into nude mice. After the LNCaP tumors were established, the NE mouse prostate allograft (NE-10) was implanted on the opposite flank of these nude mice to test whether NE tumor-derived systemic factors can influence LNCaP growth. Mice bearing LNCaP tumors with or without NE allografts were castrated 2 weeks after NE tumor inoculation, and changes in LNCaP tumor growth rate and gene expression were investigated. After castration, LNCaP tumor growth decreased in mice bearing LNCaP tumors alone, and this was accompanied by a loss of nuclear androgen receptor (AR) localization. In contrast, in castrated mice bearing both LNCaP and NE-10 tumors, LNCaP tumors continued to grow, had increased levels of nuclear AR, and secreted prostate-specific antigen. Therefore, in the absence of testicular androgens, NE secretions were sufficient to maintain LNCaP cell growth and androgen-regulated gene expression in vivo. Furthermore, in vitro experiments showed that NE secretions combined with low levels of androgens activated the AR, an effect that was blocked by the antiandrogen bicalutamide. Because an increase in AR level has been reported to be sufficient to account for hormone refractory prostate cancers, the NE cell population ability to increase AR level/activity can be another mechanism that allows prostate cancer to escape androgen ablation therapy.

ABSTRACT
Increases in neuroendocrine (NE) cells and their secretory products are closely correlated with tumor progression and androgen-independent prostate cancer. However, the mechanisms by which NE cells influence prostate cancer growth and progression, especially after androgen ablation therapy, are poorly understood. To investigate the role of NE cells on prostate cancer growth, LNCaP xenograft tumors were implanted into nude mice. After the LNCaP tumors were established, the NE mouse prostate allograft (NE-10) was implanted on the opposite flank of these nude mice to test whether NE tumor-derived systemic factors can influence LNCaP growth. Mice bearing LNCaP tumors with or without NE allografts were castrated 2 weeks after NE tumor inoculation, and changes in LNCaP tumor growth rate and gene expression were investigated. After castration, LNCaP tumor growth decreased in mice bearing LNCaP tumors alone, and this was accompanied by a loss of nuclear androgen receptor (AR) localization. In contrast, in castrated mice bearing both LNCaP and NE-10 tumors, LNCaP tumors continued to grow, had increased levels of nuclear AR, and secreted prostate-specific antigen. Therefore, in the absence of testicular androgens, NE secretions were sufficient to maintain LNCaP cell growth and androgen-regulated gene expression in vivo. Furthermore, in vitro experiments showed that NE secretions combined with low levels of androgens activated the AR, an effect that was blocked by the antiandrogen bicalutamide. Because an increase in AR level has been reported to be sufficient to account for hormone refractory prostate cancers, the NE cell population ability to increase AR level/activity can be another mechanism that allows prostate cancer to escape androgen ablation therapy.

INTRODUCTION
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Neuroendocrine (NE) cells are present in the normal and neoplastic prostate (1). Increases in NE phenotype and secretory products are closely correlated with tumor progression and androgen independence in prostate cancer (2, 3). NE differentiation can be found in 10–100% of prostate adenocarcinomas depending on the sample type (biopsy or prostatectomy specimen), method and extent of fixation, the antibody used, and the sensitivity of the technique applied (4). Malignant prostate neoplasia of the prostate with NE differentiation has poorer prognosis compared with cases without it (97% survival rate in 2 years; Refs. 5, 6). Histological studies reveal that NE cells are significantly more abundant in cancerous than in noncancerous tissues (7). In fact, the overall abundance of NE cells increases in proportion to the progression of prostate cancer from the early stage to advanced, hormone-resistant disease (8). Analyses of sera from hormone-dependent and clinically advanced, hormone-refractory prostate cancer patients show increased levels of NE cell markers such as chromogranin A (9–11) and neuron-specific enolase (12). Although many studies have reported NE differentiation in prostate cancer, most of these are limited to immunohistochemical analyses. The role of NE cells in prostate cancer growth and their effect on prostate cancer after androgen ablation therapy remains to be investigated in detail.

We have established a NE allograft model from a 38-week-old LPB-Tag, 12T-10 transgenic mouse (13, 14). Ventral lobe prostatic tissue from a 12T-10 transgenic mouse that contained a NE prostate carcinoma was transplanted s.c. to an athymic male mouse. The established allograft was termed NE-10 (14). To study the effect of NE cells on prostate cancer growth after androgen ablation therapy, we investigated the effects of NE-10 tumors on an LNCaP xenograft model.

We report that NE-10 tumors promoted LNCaP tumors to show increased AR expression and continued prostate cancer growth in the absence of testicular androgens. Also, NE secretions increased androgen-regulated promoter activity in LNCaP cells. Thus, the NE component of the developing prostate tumors appears to produce factors that regulate AR activity by enhancing sensitivity to lower circulating androgen levels in a ligand-dependent manner, increasing nuclear AR levels and/or directly activating the AR in a ligand-independent manner. Because an increase in AR level has been reported to be sufficient to account for hormone refractory prostate cancers (15, 16), the NE cell population ability to increase AR level/activity can be another mechanism that allows prostate cancer to escape androgen ablation therapy.

MATERIALS AND METHODS

Cell Culture and Materials. The human prostate carcinoma cell lines LNCaP and PC-3 were obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in RPMI 1640 (Life Technologies Inc.) medium containing 5% fetal bovine serum; 0.1% insulin, transferrin, and selenium; and 0.1% glutamine. PC-3 cells were cultured in RPMI 1640 containing 10% fetal bovine serum.

LNCaP with/without NE-10 Xenograft Model and Determination of Serum Prostate-Specific Antigen (PSA), Testosterone, and Dehydroepiandrosterone (DHEA) Levels. All of the animal studies were conducted in accordance with the principles and procedures outlined by the NIH guidelines and the Institutional Animal Care and Use Committee. One million LNCaP cells were inoculated s.c. with one-third volume of Matrigel (total injection volume was 50 µl; Becton Dickinson Labware, Lincoln Park, NJ) in the right flank region of 6-week-old male athymic nude mice (BALB/c strain; Harlan Sprague Dawley, Indianapolis, IN). After the primary LNCaP tumor reached 3–4 mm diameter (9 weeks), a small fragment (~50 mg) of NE tumor from the NE-10 allograft model was implanted s.c. into the opposite flank of the nude mice. Mice were separated into different groups. In the intact mice group...
(without castration), 8 mice with LNCaP tumors with or without NE tumor were studied. In the castrated group, 8 mice with LNCaP tumors with or without NE tumor were subsequently castrated via scrotal approach 2 weeks after NE tumor implantation. Tumor volume was measured weekly and calculated by the formula: Volume = 0.523 × long diameter (mm) × short diameter (mm). Serum PSA, testosterone (T), and DHEA levels were determined by enzymatic immunoassay kit (PSA; Diagnostic Systems Laboratories, Webster, TX) and radioimmunoassay kits (RIA; T and DHEA: Diagnostic Systems Laboratories), respectively, according to the manufacturer’s protocol. Data points were reported as mean value ± SD.

Mice were sacrificed 4 weeks after NE tumor implantation (2 weeks after castration). Blood samples were taken to determine the serum PSA, T, and DHEA levels. Two h before sacrifice, mice received i.p. injections of 10 µg/l 1:1000 diluted bromodeoxyuridine (BrdUrd) labeling reagent (Amersham Biosciences, Amersham) to determine the tumor cell proliferation rate. The LNCaP and NE tumor nodules were excised and either fixed in 10% buffered formalin and paraffin embedded for immunohistochemical analysis or snap frozen in liquid nitrogen and stored at −80°C for biochemical analysis.

Cell Proliferation. Cell proliferation in tumors was determined using a Cell Proliferation kit (Amersham Biosciences, Amersham), which measures BrdUrd incorporation into cellular DNA. The paraffin-embedded blocks were cut into 4-µm sections and placed on microscope slides. Slides were deparaffinized by immersing in xylene twice for 10 min each and hydrated by immersing in a series of 100%, 70%, and 50% ethanol, and one time in dH2O for 5 min each. Slides were placed in urea buffer (pH 6.0) and microwaved for 20 min and cooled at room temperature for 1 h. Then, slides were washed in PBS (pH 7.4) for 5 min. To block endogenous peroxidase, slides were immersed in 0.3% H2O2 for 30 min then washed with PBS. Anti-BrdUrd primary antibody (mouse monoclonal, used at 1:500 dilution) was added and incubated for 1 h at room temperature. Slides were washed in PBS; and antimouse IgG2 secondary antibody was added, incubated for 30 min at room temperature, and washed in PBS. One drop of 3, 3′-diaminobenzidine tetrahydrochloride and substrate/substrate mix prepared immediately before use was added to each slide, washed in dH2O, counterstained with Mayer’s hematoxylin, dehydrated, mounted, and analyzed by light microscope. Each tissue section was counted manually in four different areas to assess the BrdUrd-labeling index. The data were presented as percentage of BrdUrd labeling cells in 100 tumor cells.

Immunohistochemistry. Paraffin-embedded tissue sections of the LNCaP xenograft were stained immunohistochemically with antibodies against PSA (clone LK2H10; Santa Cruz Biotechnology, Santa Cruz, CA) and androgen receptor (AR; clone N20; Santa Cruz Biotechnology). The primary antibody was incubated at the appropriate concentration (AR: 1:1000; PSA: 1:2000) for 1 h at room temperature. The secondary antibody was incubated for 30 min, being either horseradish-peroxidase-conjugated goat antirabbit or goat antimouse (1:1000), respectively. Slides were rinsed extensively in tap water, counterstained with Mayer’s hematoxylin, and mounted.

Western Blot Analysis. Tumors from mice carrying only the LNCaP were pooled together for protein extraction. Additionally, LNCaP tumors from mice also bearing the NE tumor were pooled as a separate group for RNA extraction. Total RNAs were extracted using Trizol (Life Technologies, Inc., Grand Island, NY), and residual genomic DNA was removed by DNaseI (Invitrogen, Carlsbad, CA) treatment. The RNA was reverse transcribed using random primers and Superscript II (Life Technologies, Inc.) according to the manufacturer’s protocol. The primers used for amplification were AR 5'-ATCGAGGGGCAAGTAGG- CATC-3' (forward), 5'-AGCCCCACTGGGGGACA C-3' (reverse); for PSA were 5'-CTCTCGTGAGGGCCACTCT-3' (forward), 5'-GGCTGCTG GCTGAGGATCATCA-3' (reverse); the 18S rRNA primers 5'-CAAGAAC GAAAGTCGAGGTTTC-3' (forward), 5'-GAGCACTATAGGGCATTAC G-3' (reverse), were used as a control. Real-time PCR reactions were carried out in a 20-µl volume using a 96-well plate format, and fluorescence was detected using the Bio-Rad I-Cycler IQ real-time detection system.

Primary Culture of NE-10 Cells. NE-10 tumor tissue was cut into 1–2 mm³ pieces. The small tissue fragments were placed into 100-mm Primaria tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ), cultured in RPMI 1640 containing 5% fetal bovine serum, 10% heat-inactivated horse serum, 1% antibiotic-antimycotic (Life Technologies, Inc.), 50 µg/ml gentamicin (Life Technologies, Inc.), 1% l-glutamine, 1% sodium pyruvate, and 1 µl HEPES at 37°C, in a 5% CO2 incubator. When the explants displayed an initial outgrowth of NE cells (usually 1 week after plating), the culture medium was changed every 2 days. Fibroblast cells that contaminated the cultured NE cells were removed by differential trypsinization.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. All of the measurements were carried out in triplicate. LNCaP and PC-3 cells were plated in a 96-well plate (1 × 10⁵/well). After 24 h, the cells were treated with conditioned medium (containing NE extracts). To generate the conditioned medium containing NE secretions, RPMI 1640 (containing 5% dextran charcoal-stripped serum; 0.1% insulin, transferrin, and selenium; and 0.1% glutamine) was added to the NE cell cultured-dish. After 24 h, the medium (containing NE secretions) was harvested and replaced to targeting cells (LNCaP and PC-3 cells). RPMI 1640 (containing 5% dextran charcoal-stripped serum; 0.1% insulin, transferrin, and selenium; and 0.1% glutamine) was added to the targeting cells as the control. Dihydrotestosterone (DHT; 10⁻¹⁰ M) and/or bicalutamide (10⁻⁵ M; Casodex; a gift from Zeneca Inc., Wilmington, DE) were added to both media. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (17) was performed at 48 h after the cells that had been treated with conditioned medium. The experiments were repeated three times.

Transient Transfection Assay. The ARR2PB-Luc (18) vector (ARR2PB promoter is an androgen-responsive promoter) was used in the transfection experiments. LNCaP and PC-3 cells were plated at an initial density of 2.5 × 10⁵/well in 24-well tissue culture plates. After 24 h, the cells were transfected with Lipofectamine (Life Technologies Inc.) for 4 h according to the manufacturer’s protocol. After transfection, the cells were treated with conditioned medium (as described above). Both conditioned and control medium were tested with a dose-response curve for DHT (10⁻¹¹ - 10⁻⁸ M) with or without bicalutamide (10⁻⁵ M). The transfection efficiency was determined by cotransfecting pRL-CMV containing the Renilla luciferase reporter gene (Promega Corp., Madison, WI). Luciferase activity was determined using the Promega Corp. luciferase assay system at 24 h after transfection. The values plotted represent the mean of at least three individual samples ± SD.

Statistical and Image Analysis. Where appropriate, experimental groups were compared using Student’s two-tailed t-test, with significance defined as P < 0.05. For quantitation of immunoblot data, images were analyzed using Scion Image software, version 1.62 (Scion Corp., Frederick, MD).

RESULTS

NE-10 Tumors Maintain LNCaP Prostate Cancer Growth in Castrated Nude Mice. The effect of the NE-10 allograft on the growth of LNCaP prostate tumors in the absence of testicular androgens was studied in vivo. Six-week-old male nude mice were injected with one million LNCaP cells s.c. The NE-10 allograft was implanted when LNCaP tumor size reached a diameter of 3–4 mm (9 weeks). Tumor volume was measured once weekly. There was no significant difference in LNCaP tumor size after the implantation of NE tumor in intact mice (Fig. 1A). Surgical castration was performed at 2 weeks after the NE tumors were grafted. The results (Fig. 1, B and C) showed
that in mice bearing LNCaP tumor alone, tumor volume decreased by 21% compared with intact mice within 2 weeks after castration. In mice bearing the LNCaP with the NE-10 tumor, the LNCaP tumor continued to grow after castration, and LNCaP tumor volume increased 26% compared with intact mice by 2 weeks after castration. After castration, LNCaP tumor volume was 2.3-fold larger in mice bearing the LNCaP with the NE-10 tumor than that of mice bearing the LNCaP tumor alone ($P < 0.05$; Student's t test; Fig. 1B). These findings suggest that NE tumors stimulate the continued growth of the LNCaP tumor despite the removal of testicular androgens.

To additionally assess the role of NE-10 tumor on the LNCaP prostate cancer cell growth, we determined the proliferation rate of LNCaP cells in the xenograft model by BrdUrd labeling (Fig. 2, A and B). Our results showed that in castrated mice bearing LNCaP tumor alone, only 15 ± 8.6 cells per 100 LNCaP cells were stained by BrdUrd, whereas 69.5 ± 15.4 cells per 100 LNCaP cells were stained in castrated mice bearing LNCaP with NE-10 tumor (Fig. 2C). The number of LNCaP cells stained by BrdUrd in LNCaP tumor from mice bearing LNCaP with NE-10 tumor was significantly higher than mice bearing LNCaP tumor alone ($P < 0.01$; Student's t test), implying that NE cells secreted factors that were sufficient for LNCaP tumor proliferation in the absence of testicular androgens.

**NE-10 Tumor Promotes PSA Expression in LNCaP Xenografts.**

PSA gene expression is regulated by androgens. Therefore, serum PSA levels in castrated mice were measured to determine whether PSA gene expression was influenced by the presence of NE tumors alone. Our results showed that in mice bearing both LNCaP and NE-10 tumors, serum PSA levels (Fig. 3) increased 1.4-fold compared with mice bearing LNCaP tumors alone. In the castration group, for the mice bearing both LNCaP and NE-10 tumors, serum PSA levels increased 2.1-fold compared with mice bearing LNCaP tumors alone. Immunohistochemical analysis showed that higher levels of PSA staining were detected in LNCaP xenograft tissue when the NE-10 tumor was present (Fig. 4A) compared with that in LNCaP tumors alone in castrated mice (Fig. 4B).

To additionally determine whether the NE tumor promoted PSA expression in LNCaP cells, total protein and RNA were extracted from the LNCaP xenograft (from castrated mice groups). Western blot analysis determined (Fig. 5A) that PSA protein levels in the LNCaP xenograft tissue from mice bearing both LNCaP and NE-10 tumors were 1.3-fold higher than when the NE-10 tumor was absent (Fig. 5B). In addition, real-time RT-PCR analysis measuring PSA expression also showed that PSA expression from LNCaP xenografts from mice with both LNCaP and NE-10 tumors increased 2.2-fold over that in mice carrying LNCaP tumor alone (Fig. 5C).

To understand whether NE tumors induce changes in androgen levels, serum T and DHEA levels were measured. Our results showed that serum T and DHEA levels have no statistical difference between the groups of LNCaP plus NE-10 tumors and LNCaP tumors alone. These levels are the same as those seen in nude mice not bearing any grafts (Table 1). However, after castration the T levels decreased compared with intact controls such to values below the sensitivity (<0.1 ng/ml) of the assay, whereas DHEA levels did not change (Table 1). These results suggest that NE-10 tumors have no significant effects on the serum androgen (T and DHEA) levels.

Collectively, the findings showed that NE-10 tumors promote PSA secretion and expression at the protein and mRNA levels in LNCaP xenograft but no detectable changes in serum T and DHEA levels.

**NE-10 Tumor Increases Expression of AR in LNCaP Xenograft.**

LNCaP cell growth is androgen dependent. Increased proliferation as well as PSA expression is mediated through the AR. The Western blot analyses of LNCaP tumors grown in the presence of NE-10 tumors (castrated mice) showed (Fig. 5A) that both AR and KFBP5.1, an AR regulated gene, were 2.45- and 3.18-fold higher, respectively, than that in the LNCaP tumors grown in the absence of NE-10 (Fig. 5B). Also, the real-time RT-PCR determined that AR expression in LNCaP tumors was 1.7-fold higher when the NE-10
tumor was present (Fig. 5C). In addition, immunohistochemical analysis suggests that AR staining was more intense in nuclei from LNCaP xenograft tissue in presence of NE-10 tumor compared with that when NE-10 tumors were absent (Fig. 4, C and D). Together, these findings suggest that the presence of NE-10 tumors stimulates LNCaP tumor growth, and increases AR expression, PSA expression, and PSA secretion in castrated mice.

**NE-10 Cell Secretions Promote the Growth of LNCaP Cells in Vitro.** To test the hypothesis that NE-10 tumor secretions promote prostate cancer cell growth through AR, AR-positive LNCaP cells and AR-negative PC-3 cells were treated using conditioned culture medium containing NE cell secretions (see “Materials and Methods”). Our results demonstrated that, after 48 h of treatment, in the absence of androgen, the numbers LNCaP cells that cultured in with or without NE-10 cell secretion media decreased 35% and 38%, respectively, compared with the starting cell number. However, in the presence of androgen (10^{-8} M DHT), the number of LNCaP cells increased by 214% when NE-10 cells secretions were present and 141% when NE-10 cells secretions were absent compared with the starting cell number (Fig. 6A). The number of LNCaP cells that cultured in conditioned medium was significantly higher (P < 0.01; Student’s t test) than that of the cells cultured in medium without NE secretions in the presence of androgen. Furthermore, bicalutamide (10^{-5} M), an antiandrogen, blocked this effect (Fig. 6A). To additionally assess whether this response was due to the presence of AR, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was repeated in PC-3 cells, which do not express AR. As shown in Fig. 6B, PC-3 cell growth rate did not change in any of the conditions tested. These results suggest that NE-10 cell secretions promote the growth of LNCaP cells based on the presence of androgen. Therefore, the effect of NE cells on the prostate cancer growth may be mediated through effects on the AR.

**NE Enhances the Functional Activity of AR in the Presence of Androgen.** Because our results indicate that NE-10 secretions promote androgen-induced LNCaP cell growth, the following experiments performed to test whether NE-10 secretions also enhance androgen-regulated transcription. The ARR2 PB-Luc vector was transfected into LNCaP and PC-3 cells, and the cells were treated with/without NE secretion containing medium. In the absence of androgen, ARR2 PB promoter had no activity in LNCaP cells either with or without NE secretions. However, in the presence of androgen (10^{-8} M DHT), the activity of ARR2 PB promoter was significantly increased in the presence of NE secretions, but not in the absence of NE secretions. These results suggest that NE-10 cell secretions enhance the functional activity of AR in the presence of androgen.

**Fig. 2. NE-10 tumor maintains LNCaP cells proliferation in castrated mice.** Two h before being sacrificed, the mice received an i.p. injection of bromodeoxyuridine (BrdUrd; BrdUr) labeling reagent to determine tumor cell proliferation. A, LNCaP tumor from mice model bearing LNCaP tumor alone. B, LNCaP tumor from mice model bearing LNCaP with NE-10 tumor. Each tissue section was counted manually in four different areas to assess the BrdUrd-labeling index. The data were then presented as number of BrdUrd-labeling cells (arrows) in 100 tumor cells. Results are expressed as the mean number; bars, ± SD. **P < 0.01 by Student’s t test, compared with control (without NE-10 tumor).

**Fig. 3. NE-10 tumor increases serum prostate-specific antigen (PSA) level in LNCaP xenograft mice.** At the end of the experiments, blood samples were taken and serum PSA levels were determined by enzymatic immunoassay. The results were reported as mean value; bars, ± SD. *, P < 0.05 by Student’s t test, compared with control (without NE-10 tumor).
higher (P < 0.01; Student’s t test) when treated with NE secretions containing medium then that when NE secretions were absent in the medium at these same concentrations of androgens. Very low levels of androgens (10^(-11) and 10^(-10) M DHT) did not show significant effects when they were combined with NE secretions (Fig. 7A). The reduction by androgen was blocked by bicalutamide (10^(-5) M), an AR blocker (Fig. 7A). In AR-negative PC-3 cells, the ARR-PB promoter had no induced activity with conditioned medium, irrespective of DHT and/or bicalutamide stature (Fig. 7B). These findings suggested that NE secretions increase the functional activity of AR in the presence of androgen. Collectively, NE tumor affects the growth of prostate cancer through the ligand-dependent AR signaling pathway.

**DISCUSSION**

Many studies suggest that in prostate cancer, NE differentiation is closely correlated with tumor progression and androgen independence. However, mechanisms by which NE secretory factors affect prostate cancer growth to promote androgen-independent progression after androgen ablation therapy are poorly understood. In this study, we investigated the role of NE tumor cells on prostate tumor growth both *in vivo* and *in vitro*. Our results demonstrated that NE cancers promote LNCaP tumor growth in castrated mice. After castration, only very low levels of T from the adrenal gland and higher levels of weak androgens such as DHEA would be present. Our data suggest that the effect of NE cells on LNCaP cells may be mediated through increased sensitivity of prostate cancer cells to androgens, increased AR expression, and/or by NE tumor secretory factors. This conclusion is drawn from the following observations in castrated mice: (a) NE-10 tumors produce factors that systemically act to maintain LNCaP tumor growth; (b) NE-10 tumors increased AR expression in LNCaP xenografts; (c) NE-10 tumors increased the expression of PSA and KFBP5.1, two AR-regulated genes in LNCaP xenografts; and (d) NE-10 tumors did not induce any changes of serum T and DHEA

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* DHEA, dehydroepiandrosterone; NE, neuroendocrine.
levels in nude mice. Furthermore, in vitro experiments also support these conclusions. First, NE-10 secretions had no effect on the growth rate of LNCaP cells in the absence of androgen in vitro. However, the combination of low concentrations of androgens and NE secretions synergistically promotes the growth of LNCaP cells. This effect was blocked by the antiandrogen bicalutamide (an antiandrogen that does not activate the mutated AR of LNCaP cells), suggesting that the mitogenic effect of NE secretions on prostate tumor growth is dependent on AR. Secondly, transient transfection assays in LNCaP cells using the androgen-responsive ARR2 PB-luciferase construct showed that NE-10 secretions enhanced DHT-activated reporter gene activity. Bicalutamide also blocked this effect. Lastly, in AR-negative PC-3 cells, NE-10 secretions did not stimulate cell proliferation and did not enhance the activity of ARR2 PB promoter. Although it has been suggested the NE cells may promote prostate cancer cell growth, to our knowledge, this is the first report that demonstrates that NE prostate cancer cells do promote prostate cancer growth in vivo.

Several studies have suggested that NE differentiation in carcinoma of the prostate appears to be associated with poor prognosis, tumor progression, and the androgen-independent state. NE differentiation in androgen-independent tumors appears to increase when compared with androgen-dependent tumors, as determined by immunohistochemistry (19, 20), chromogranin A plasma levels, and chromogranin A gene expression (11, 21). It has been suggested that NE differentiation of prostate cancer cells can be induced by androgen withdrawal (22). NE differentiation in prostate adenocarcinoma is usually manifested as isolated foci or islands of cells expressing certain NE-related products (23). Using double label techniques in human tissue, Bonkhoff et al. (5, 6) reported that increased proliferation in exocrine cells surrounded NE tumor cells. Peptide growth factors and cytokines, operating either within the context of androgen-dependent signaling mechanisms or in an androgen-independent context, are likely to be critical to the process of prostate cancer progression. Our experiments demonstrated that NE tumors increased LNCaP tumor growth by secreting factors that can systemically act on a distant site.

AR is a major regulatory transcription factor in normal prostate growth and development and in the growth of androgen-dependent prostate cancer. In patients undergoing androgen ablation therapy, a role for AR-mediated gene activation in recurrent prostate cancer is supported by its expression together with the expression of androgen-regulated genes (24–26). Our data strongly suggest that NE differentiation is an important mechanism by which prostate cancer can become “androgen refractory.” This mechanism could work synergistically with other proposed mechanisms that activate AR-induced signaling pathways, such as AR reactivation in recurrent prostate cancer that include altered growth factor-induced phosphorylation (27–31); AR mutations (32) that broaden ligand specificity (33); AR gene amplification, which was observed after androgen deprivation in 30% of recurrent prostate cancers (34); and AR overexpression, which is associated with increased sensitivity to the growth-stimulating effects at low androgen concentrations in recurrent prostate cancer.

Fig. 6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated the effect of NE secretions on the growth of LNCaP (A) and PC-3 (B) cells in vitro. The MTT assay was performed 48 h after treatment with or without conditioned media. The number of cells was presented as relative cells number (%) compared to the before treatment based on the OD540 value measured by MTT assay. The results were reported as mean value (%); bars, ±SD. **, P < 0.01 by Student’s t test; W/O, without; DHT, dihydrotestosterone.

Fig. 7. Transient transfection assay of ARR 2 PB-Luc demonstrated the effect of NE secretions on the activity of androgen receptor. LNCaP (A and B) and PC-3 (C) cells were transfected with the ARR 2 PB-luciferase construct together with the pRL-CMV construct to control for transfection efficiency. All of the assays measured triplicate samples. The firefly activity was normalized for luciferase activity and protein content. The firefly activities of samples were showed as the mean; bars, ±SD. **, P < 0.01 by Student’s t test compared with control (without NE secretions); W/O, without; DHT, dihydrotestosterone.
derived cell lines (35, 36). Koh et al. (37) reported that adrenal steroids, such as DHEA, can be converted into DHT in LNCaP cells. Thus, it is possible that the weak adrenal steroids may work in concert with the NE secretions. Also, human prostate tissue can convert DHEA to active androgens (38). In this study, we have demonstrated that in castrated mice, NE-10 tumors increased AR and AR-regulated gene expression in LNCaP xenografts with no detectable changes in serum androgen (T and DHEA) levels. Consistent with a recent report (15, 16), our data suggest that NE cells increased the sensitivity of prostate cancer cells to the androgen by an increase of the AR levels. However, we cannot exclude the possibility that the NE-10 graft also alters intratumor metabolism of DHEA to T by LNCaP cells.

To understand how the NE cells promote AR-induced transcription, we investigated the effects of NE secretions on ARR2PB, an androgen-responsive promoter. The results showed that NE secretions enhanced the ARR2PB activity, even in the presence of lower androgen levels (10⁻⁷ m, DHT) and this effect could be blocked by the antiandrogen bicalutamide. NE secretions, furthermore, stimulated the proliferation of prostate cancer cells in vitro in the presence of androgen, which could be blocked by bicalutamide. Taken together, these findings indicate that the effects of NE cells on prostate cancer growth are mediated through increased activity of functional AR. Therefore, yet to be defined neuropeptides may play an important role in promoting the transition of prostate cancer from an androgen-dependent to androgen-independent state during androgen-deprivation therapy.

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REFERENCES

NE-10 Neuroendocrine Cancer Promotes the LNCaP Xenograft Growth in Castrated Mice

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