Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells

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Abstract

Neuroendocrine (NE) cells are the minor cell populations in normal prostate epithelial compartments. During prostate carcinogenesis, the number of NE cells in malignant lesions increases, correlating with its tumorigenicity and hormone-refractory growth. It is thus proposed that cancerous NE cells promote prostate cancer (PCa) cell progression and its androgen-independent proliferation, although the origin of the cancerous NE cells is not clear. To investigate the role of cancerous NE cells in prostate carcinogenesis, we characterized three NE subclone cell lines–NE-1.3, NE-1.8 and NE-1.9, which were transdifferentiated from androgen-sensitive human PCa LNCaP cells by culturing in an androgen-depleted environment, resembling clinical androgen-ablation therapy. These subclone cells acquire many features of NE cells seen in clinical prostate carcinomas, for example exhibiting a neuronal morphology and expressing multiple NE markers, including neuron-specific enolase, chromogranin B, neurotensin, parathyroid hormone-related peptide, and to a lesser degree for chromogranin A, while lacking androgen receptor (AR) or prostate specific antigen (PSA) expression. These cells represent terminally differentiated stable cells because after 3 months of re-culturing in a medium containing androgenic activity, they still retained the NE phenotype and expressed NE markers. Despite these NE cells having a slow growth rate, they readily developed xenograft tumors. Furthermore, media conditioned by these NE cells exhibited a stimulatory effect on proliferation and PSA secretion by LNCaP cells in androgen-deprived conditions. Additionally, we found that receptor protein tyrosine phosphatase plays a role in upregulating multiple NE markers and acquiring the NE phenotype. These NE cells thus represent cancerous NE cells and could serve as a useful cell model system for investigating the role of cancerous NE cells in hormone-refractory proliferation of PCa cells.

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Introduction

In the normal mature prostate, neuroendocrine (NE) cells are distributed along the epithelial compartment in the prostate gland (di Sant’Agnese & de Mesy Jensen 1984). These NE cells contain dense core granules in cytoplasm and secrete several neuronal markers as well as growth-stimulatory factors, while they lack the expression of androgen receptor (AR). It is proposed that these NE cells originate from...
endodermal epithelial cells and are terminally differentiated (Cussenot et al. 1994). These NE cells may play a functional role in regulating the homeostasis in the growth and differentiation processes of normal prostate epithelial cells in an androgen-independent manner (Sciarra et al. 2003).

The role of cancer-associated NE cells in prostate cancer (PCa) progression has received much attention in both basic and clinical studies recently. Several lines of evidence suggest that NE differentiation is a common feature of prostate carcinogenesis (di Sant’Agnese 1992) and is often associated with tumor progression and poor prognosis (Abrahamsson 1999). For example, as seen in archival clinical specimens, the population of NE cells, both in terms of their numbers and as a percentage, increases, especially in hormone-refractory tumors (Jiborn et al. 1998, Ito et al. 2001, Ismail et al. 2002, Hirano et al. 2004). PCa that are composed solely of NE cells, for example small cell carcinomas and carcinoid tumors, are associated with high aggressiveness (Abrahamsson 1999). Since NE cells secrete a variety of growth factors, cancerous NE cells may promote the proliferation of adjacent PCa cells via a paracrine mechanism in an androgen-ablated environment (Abrahamsson 1999). This notion is supported by the observations in clinical specimens that cancer cells adjacent to NE cells exhibit higher proliferation indices than distant cancer cells (Bonhoff et al. 1995). Furthermore, the level of NE markers such as neuron-specific enolase (NSE), Chromogranin (Cg)A, and CgB may be elevated in the circulation of advanced hormone-refractory PCa patients, compared with the early-stage cancer patients and may serve as a surrogate marker for advanced tumor progression (Kadmon et al. 1991, Tarle & Rados 1991). Thus, it is imperative to identify the origin of these cancerous NE cells, to analyze the mechanisms underlying the development of these cells and to investigate their role in PCa progression for improving the treatment of this cancer.

Results of several studies show that PCa cells can acquire the NE-like phenotype upon various treatments in culture. For example, following exposure to epinephrine and forskolin that increase the intracellular level of cAMP, LNCaP and PC-3 cells acquire the NE phenotype and express neuronal markers (Bang et al. 1994, Cox et al. 1999). Cytokines, for example interleukin-6 (Qiu et al. 1998, Mori et al. 1999) and interleukin-1β (Diaz et al. 1998), and growth factors, for example heparin-binding epidermal growth factor (Kim et al. 2002), can also induce LNCaP cell transdifferentiation. Furthermore, in a steroid-reduced (SR) environment, LNCaP cells acquire the NE-like phenotype and express NE markers as that observed in clinical specimens from PCa patients under androgen-ablation therapy (Burchardt et al. 1999, Zelivianski et al. 2001). Although those studies showed interesting results regarding NE differentiation of PCa cells, the information, for example about the expression of NE markers in those cells, is limited. Importantly, those transdifferentiation processes are only transient and cells fully revert to their original phenotype in the absence of inducers (Burchardt et al. 1999, Cox et al. 1999, Ismail et al. 2002). As such, they differ from those terminally differentiated clinical cancerous NE cells and they cannot be used for studying the interaction between cancerous NE and PCa cells. To elucidate the molecular mechanism of NE transdifferentiation and their role in PCa progression, stable NE cell lines of clinical relevance are needed.

Receptor protein tyrosine phosphatase α (RPTPα), a member of the transmembrane subfamily of protein tyrosine phosphatases (PTPs), is widely expressed in mammalian tissues (Sap et al. 1990). Among several molecules that could be associated with RPTPα function, c-Src tyrosine kinase is its best-characterized substrate (den Hertog et al. 1993, 1994, Zheng et al. 2000, Stetak et al. 2001). In RPTPα-null cells, for example, the specific activity of c-Src protein is greatly reduced and can be restored by ectopic expression of RPTPα (Ponniah et al. 1999, Su et al. 1999). This is because RPTPα, via its phosphorylated Tyr-789 residue, interacts with the Src homology 2 (SH2) domain of c-Src, which results in the dephosphorylation of c-Src at Tyr-527 (Zheng et al. 2000), the negative regulatory site of c-Src, and leads to the activation of c-Src by phosphorylation at Tyr-418, the positive regulatory site. RPTPα is involved in a variety of cellular functions, including neuronal differentiation (den Hertog et al. 1993, van Inzen et al. 1996), the regulation of insulin signaling in prolactin gene expression (Moller et al. 1995, Jacob et al. 1998) and integrin-mediated cell-substratum adhesion (Su et al. 1999). In PCa cells, RPTPα has a very low level of expression (Zelivianski et al. 2000), while its expression is increased in SR conditions, correlating with NE differentiation (Zelivianski et al. 2001). Despite the involvement of RPTPα in neuronal differentiation in several cell types, its role in prostate NE differentiation requires further investigation.

In this report, we describe the establishment and the biochemical properties of three NE subclones, including NE-1.3, NE-1.8, and NE-1.9 cells. These NE cells were transdifferentiated from androgen-sensitive LNCaP cells by culturing in an SR environment, mimicking the androgen-ablation condition in the
clinical setting. Importantly, these cells represented the cancerous NE cells because they are terminally differentiated, lack AR and prostate-specific antigen (PSA) expression, express multiple NE markers, and retain some epithelial characteristics, as seen in clinical PCa. In addition, the media conditioned by those NE cells stimulated the proliferation and the PSA secretion by androgen-sensitive LNCaP cells in an SR environment.

Materials and methods

Materials

Gentamicin, l-glutamine, Phenol Red-positive or -negative RPMI 1640 medium, OPTI-MEM-I medium, Lipofectamine and Plus reagents, as well as mammalian expression vector pTracer-CMV2, were purchased from Invitrogen (Carlsbad, CA, USA). Certified charcoal/dextran-treated fetal bovine serum (FBS) with a testosterone concentration of less than 0.1 nM was from Hyclone (Logan, UT, USA). SDS, glycine, acrylamide/bisacrylamide (37.5 : 1) solution, Coomassie Blue-based protein-assay reagent, and prestained and unstained standard protein markers were obtained from Bio-Rad (Hercules, CA, USA). The antibody against the first phosphatase domain of human RPTPα was provided by Dr Parminder P. Mehta (University of Nebraska Medical Center, Omaha, NE, USA). Normal goat serum was purchased from Vector Laboratories (Burlingame, CA, USA). The Super Signal West Pico chemiluminescent reagent was from Pierce (Rockford, IL, USA). PP2 and PD98059 were from Calbiochem (San Diego, CA, USA). Other reagents were from Sigma unless indicated otherwise. The porous culture inserts (0.4 μm) were from BD Falcon (Franklin, NJ, USA).

Cell culture and cDNA transfection

Human prostate adenocarcinoma LNCaP and PC-3 cells were originally obtained from American Type Culture Collection (Manassas, VA, USA). LNCaP and PC-3 cells were routinely maintained in the regular medium (Phenol Red-positive RPMI 1640 medium supplemented with 5% FBS, 1% glutamine, and 0.5% gentamicin) as described previously (Lin et al. 1992, Zelivianski et al. 2001). NE-1.3, NE-1.8, and NE-1.9 cells were maintained in an SR medium (Phenol Red-free RPMI 1640 medium supplemented with 5% charcoal/dextran-treated, heat-inactivated FBS, 1% glutamine, and 0.5% gentamicin; Zelivianski et al. 2001, Zhang et al. 2003). For experiments, androgen-sensitive LNCaP cells with passage numbers 28–33 were utilized (Lin et al. 1998). For growth analysis, cells were seeded at a density of 4 × 10^4 cells per well in six-well culture plates in the corresponding medium. After 3 days, one set of attached cells was harvested and counted as day 0 with a Z1 model Coulter Counter (Coulter Corporation, Miami, FL, USA) and the remaining cells were fed with fresh medium. The total cell numbers were then counted on days 3, 6, and 9, while fresh medium was added to the remaining cells on day 3 and 6. For cDNA transfection, cells were plated in six-well plates or 60 mm culture dishes. After 48-h incubation, cells were transfected with appropriate amounts of cDNAs by liposome-mediated transfection reagents. Forty-eight hours after transfection, cells were harvested for experiments.

Microsatellite DNA analyses

Genetic analyses of both NE-1.3 and NE-1.8 cells were performed with simple sequence repeat microsatellite DNA markers using PCR, as described previously (Karan et al. 2001, Igawa et al. 2002). The amplified PCR products were denatured and fractionated on 6% polyacrylamide gels, containing 7 M urea. The gels were dried and exposed to X-ray film to analyze the genetic profile of NE-1.3 and NE-1.8 cells, compared with LNCaP cells. Genomic DNA from PC-3 cells, pancreas, benign prostate hyperplasia (BPH), or tracheal tissues were used as controls.
Immunoblotting

Cells were rinsed twice with ice-cold HEPES-buffered saline, pH 7.0, scraped, and lysed in an ice-cold cell lysis buffer containing protease and phosphatase inhibitors, as described previously (Lin et al. 1998). The lysates were centrifugated at 1600g for 10 min at 4°C. The protein concentration of the supernatant was determined using a Bio-Rad protein-assay kit. For immunoblotting, an aliquot of total cell lysates (50–100μg) in SDS sample buffer was electrophoresed and transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, the membrane was incubated with the primary antibody for 3 h at room temperature or overnight at 4°C. After rinsing to remove the excess antibody, the membrane was incubated with an appropriate secondary antibody for 1 h at room temperature. The specific protein was detected by an ECL reagent kit.

**Effects of NE CM on cell proliferation and PSA secretion**

To collect conditioned medium (CM), 1×10⁶ NE-1.3, NE-1.8, or LNCaP cells were seeded in T25 culture flasks in SR or regular media. After 3 days, cells were fed with 7ml fresh corresponding medium and maintained for 5 days. CM was collected and centrifugated at 2000g for 10 min at 4°C. The pH of supernatant was adjusted to 7.0 and the medium was filtered through a 0.2μm nylon filter. Different concentrations of CM were prepared as indicated in each set of experiments. For experiments, LNCaP cells were seeded in six-well plates at 5×10⁴ cells per well in regular medium and allowed to attach for 48h. Subsequently, cells were fed with the SR medium and incubated for an additional 48h. One set of attached cells was then counted as day 0 and the remaining cells were fed with fresh SR medium containing different concentrations of CM by LNCaP or NE cells. Cells were harvested and counted every 2–3 days and the fresh corresponding SR-CM was added to the remaining cells on the same day. To analyze the effect of NE CM on the PSA secretion, 5×10⁵ LNCaP cells were plated on T25 culture flasks in the regular medium for 48h. The attached cells were fed with the SR medium and incubated for an additional 48h. Subsequently, cells were fed with 5% CM by NE cells. The control cells were fed with the SR-medium alone. After a 3-day incubation, the media were collected for analyzing the secreted level of PSA.

For co-culturing experiments, LNCaP cells were plated into porous inserts (0.4μm) with 3×10⁴ cells/insert in six-well plates and maintained in the regular culture medium. The following days, LNCaP and NE-1.3 cells were seeded, respectively, into six-well plates with 3×10⁵ cells/well in the regular culture medium or the SR medium. After 3 days, the medium in the inserts was changed to SR medium and placed into wells that were seeded with LNCaP or NE-1.3 cells in the corresponding medium. After a 5-day incubation, the number of LNCaP cells in each insert were counted.

**Immunofluorescence staining**

Cells were plated on glass coverslips and maintained in corresponding conditions for 72 h. After washing with PBS, cells were fixed with 2% paraformaldehyde and permeabilized by 0.2% Triton X-100 in PBS for 30 min. After blocking with normal goat serum, fixed cells were incubated with primary antibody for 1 h at room temperature. Control staining was performed without primary antibody. After three washes with PBS containing 0.25% Tween 20, cells were incubated for 30 min at room temperature with secondary antibody, Alexa 594-conjugated goat anti-mouse or anti-rabbit IgG. Fluorescence was visualized with a Nikon ECLIPSE E800 fluorescence microscope or the Zeiss LSM 410 confocal laser scanning microscope (Zeiss, Goettinger, Germany) with 400× magnification at the University of Nebraska Medical Center (UNMC) Confocal Microscope Core Facility.

**Effect of RPTPα expression on cell morphology**

To determine the effect of RPTPα on inducing NE-like morphology, LNCaP cells were plated on glass coverslips and co-transfected with 0.25μg green fluorescent protein (GFP) expression vector pTrace-CMV2 plus 1μg pSG5-RPTPα plasmid or pSG5 vector alone. After transfection, cells were maintained in the regular medium for 48h and then fixed with 2% paraformaldehyde. The morphology of GFP-positive cells was visualized with a Nikon ECLIPSE E800 fluorescence microscope.

**Analysis of tumorigenicity**

To analyze the clonogenic growth activity of NE subclone cells, cells were seeded in six-well plastic plates with SR medium at densities of 50, 500, or 5000 cells per well. LNCaP parental cells plated in the regular medium were used as a control. After 24 h, the attached cells were fed with corresponding fresh medium and maintained for an additional 9 days with the addition of 2ml of fresh medium per well on days 4.
and 7. On day 10, the attached cells were stained with 0.1% Crystal Violet solution.

For the xenograft animal model experiment, the protocol was approved by the IACUC Committee at UNMC. Each group of three male athymic BALB/C null/null mice at 8 weeks old (Charles River Laboratory, Wilmington, MA, USA) was injected subcutaneously with $2 \times 10^6$ LNCaP parental cells or $1 \times 10^6$ NE-1.3 subclone cells with Matrigel (1:1, v/v; Collaborative Biochemical Products, Bedford, MA, USA) in a final volume of 0.2 ml per mouse. Tumors were measured once per week with calipers, and tumor volumes were calculated as described previously (Igawa et al. 2002).

Results

Establishment of NE cell lines

To investigate the role of cancerous NE cells in PCa progression, we established stable NE cells by culturing androgen-sensitive LNCaP cells in an androgen-reduced condition, resembling clinical androgen ablation therapy. LNCaP cells were initially seeded in the regular culture medium for 3 days and then continuously maintained in an SR medium. Under this condition, the cell growth diminished and the morphology gradually changed into a NE-like phenotype (Fig. 1A). The population of NE-like cells was maintained in the SR medium and the single cell was isolated using a glass cylinder and propagated. After approximately 6 months, over 20 subclone cells grew and became independent cell lines. Three stable cell lines, designated NE-1.3, NE-1.8, and NE-1.9 (Fig. 1A), were randomly chosen for biochemical characterizations. LNCaP cells maintained in the regular medium were used as a control, while LNCaP-SR cells (LNCaP cells cultured in an SR condition for 2 weeks) were used as another control. As shown in Fig. 1A, LNCaP cells have an epithelial morphology, tapering into unbranched processes, in general, shorter than the cell body. LNCaP-SR cells, as well as three NE cell types, exhibited a neuronal morphology with compactly rounded cell bodies, having extended and fine branched processes (Fig. 1A). Thus androgen-sensitive LNCaP cells acquired a NE-like phenotype in an SR condition.

Genetic characterization of NE cells

To analyze the genetic profile of NE cells, genomic DNA from NE-1.3 and NE-1.8 cells were used for microsatellite DNA analyses with the simple sequence repeat microsatellite DNA markers. As shown in Fig. 1B, of the four markers examined, both NE cell types exhibited the same microsatellite DNA markers as LNCaP cells. As controls, PC-3 cells, BPH, pancreatic, and tracheal tissues exhibited different markers from LNCaP and NE cells, with the exception of the D5S 428 marker, which was uninformative (Fig. 1B). These results indicate the genetic lineage of NE-1.3 and NE-1.8 cells from LNCaP cells.

Expression of NE markers and growth factors in NE cells

We analyzed the expression profile of NE markers in those three NE cell types and it compared with that of LNCaP parental and LNCaP-SR cells. As shown in Fig. 2A, LNCaP cells expressed a low level of NSE, a classical marker of neuronal cells (Carlei et al. 1984), while LNCaP-SR cells showed increased levels of NSE and three NE cell types had very high levels of NSE, over 20-fold higher than LNCaP cells. Furthermore, all three NE cell types expressed approximately 5–10-fold higher levels of CgB, but not CgA, than LNCaP cells (Fig. 2A). Immunoblot analyses further showed that the expression level of PTHrP, a growth factor of NE cells (Iwamura et al. 1995, Asadi et al. 1996), was very low in LNCaP cells, while its expression level was greatly increased in all three NE cell types (Fig. 2A). Additionally, the three NE cell types expressed a high level of NT protein, another NE marker and growth stimulator, but not LNCaP or LNCaP-SR cells (Fig. 2A). Interestingly, LNCaP-SR and the three NE cell types expressed elevated levels of keratin 8 and 18, two types of intermediate filament that serve as prostate epithelial-differentiation markers (Zi & Agarwal 1999), higher than LNCaP parental cells (Fig. 2A). Furthermore, western blotting and immunocytochemical staining revealed that the expression level of Bcl-2, an anti-apoptotic protein, was increased in LNCaP-SR cells and highly elevated in all three NE cell types, higher than that in LNCaP parental cells (Fig. 2A and B). The data collectively showed that those three NE cell types express high levels of multi-markers, including NSE, CgB, PTHrP and NT, keratin 8/18 and Bcl-2, while CgA was elevated only in NE-1.9 cells.

Expression of AR, PSA and PAcP in NE cells

We investigated the expression of AR and two prostate epithelium-differentiation antigens, PSA and PAcP, in NE subclone cells. Results of immunocytochemical staining clearly showed that LNCaP cells have strong staining for AR and PSA, while all three NE cell types only exhibited weak background staining (Fig. 2B).
Western blot analyses further validated that three NE cell types did not express a detectable level of AR and PSA, even after prolonged exposure with ECL reagents (Fig. 2C). Nevertheless, all three NE cell types expressed PAcP although the level was variable (Fig. 2B and C). Thus, after long-term culturing in an androgen-depleted condition, the expression of AR and PSA was completely suppressed, but not that of PAcP.

To examine whether the altered phenotype is a reversible process as described in previous reports (Cox et al. 1999, Ismail et al. 2002), NE-1.3 and NE-1.8 cells were cultured for approximately 3 months in the

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**Figure 1** The morphology and microsatellite DNA analyses of NE cells. (A) Androgen-sensitive LNCaP cells were seeded initially in the regular medium for 3 days, and subsequently maintained in an SR medium. Three stable NE cell lines, NE-1.3, NE-1.8, and NE-1.9, were shown, comparing with LNCaP cells and LNCaP-SR cells. The morphology of those cells was visualized with an optical microscope in x200 original magnification. (B) Genomic DNA from LNCaP, NE-1.3 and NE-1.8 cells were analyzed for their genetic profiles. PC-3 cells and tissues (BPH tissues for D2S 1399, pancreatic tissues for D4S 2366 and DSS 428, tracheal tissues for D19S 591) were used as controls. Four markers, including D2S 1399, D4S 2366, D19S 591, and DSS 428, were examined. Similar results were obtained in three sets of independent experiments.
regular medium, as for LNCaP cells, which contains androgenic activity. As shown in Fig. 2D, neither AR nor PSA expression was restored in those re-cultured NE cells, while the high level of NSE was retained, similar to corresponding cells in the SR condition. Additionally, those NE cells retained their neuronal morphology (data not shown). Together, these data suggest that our NE cells are stable NE cell lines that were irreversibly transdifferentiated from androgen-sensitive LNCaP cells in an SR environment.

**Tumorigenic activities of NE cells**

To examine the tumorigenic activity of NE cells, initially, we determined their growth rates. As shown in Fig. 3A, NE-1.3 and NE-1.8 cells had slower growth rates than LNCaP parental cells.
three NE cell types had higher clonogenic growth than LNCaP parental cells (Fig. 3B). To further determine the tumorigenicity of NE subclone cells in vivo, NE-1.3 and LNCaP cells were subcutaneously inoculated with Matrigel into athymic mice, respectively. Fig. 3C showed that NE-1.3 cells developed measurable tumors in all three injected mice with sizes of approximately 100 mm$^3$ 1 week after inoculation, while only one of three LNCaP parental cell-injected mice developed a measurable tumor of approximately 50 mm$^3$ after 2 weeks. The data collectively indicated that, despite a slower growth rate in culture, NE cells exhibit higher tumorigenicity than LNCaP parental cells.

**Expression of RPTPα in NE cells**

RPTPα plays a critical role in neuronal differentiation (den Hertog et al. 1993, van Inzen et al. 1996). Androgen depletion induces RPTPα expression as well as NE differentiation of LNCaP cells (Zelivianski et al. 2001, Zhang et al. 2003). We analyzed the expression of RPTPα in NE cells. The expression of RPTPα in all three NE cell types was increased by over 5-fold compared with that in LNCaP cells (Fig. 4A). Further, the expression of RPTPα correlated with the activation of ERK1/2, as indicated by their phosphorylation levels (Fig. 4A). To clarify whether NE cells were derived from a subpopulation of LNCaP cells that express high levels of RPTPα in the SR condition, we performed immunofluorescence staining of RPTPα in NE-1.3 and NE-1.8 cells, comparing it with that in LNCaP and LNCaP-SR cells. As shown in Fig. 4B, despite minor microheterogeneities in RPTPα staining in the total population of LNCaP cells, the expression of RPTPα was in general very low. RPTPα was elevated in LNCaP-SR cells, and a very high level of staining was seen in NE-1.3 and NE-1.8 cells (Fig. 4B). Together, the data indicated that androgen depletion induces NE transdifferentiation of androgen-sensitive LNCaP cells, which correlates with an elevated level of RPTPα.

**Effect of RPTPα signaling on the expression of NE markers in LNCaP cells**

Since increased expression of RPTPα (Fig. 4A) correlates with elevated levels of multi-marker proteins in NE cells (Fig. 2A), we investigated the role of RPTPα in the upregulation of those markers. We also determined whether its signal pathway is mediated by c-Src and ERK1/2 (Zheng & Pallen 1994). LNCaP cells were transiently transfected with cDNAs encoding the wild-type or the Y789F mutant of RPTPα, a mutant that blocks c-Src-mediated signaling (Zheng et al. 2000), and followed by analyzing the expression

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**Figure 3** The tumorigenic activities of NE cells. (A) Growth rate of NE-1.3 (passage 19), NE-1.8 (passage 19), and LNCaP C-33 cells. Cells were seeded and maintained as described in the Materials and methods section. The total cell number was counted on days 3, 6, and 9, and then normalized to day 0. (B) The clonogenic growth of NE cells. LNCaP (LN) and the three NE cell types were plated at different cell densities in regular and SR medium, respectively. On day 10, attached cells were fixed and stained with Crystal Violet solution. (C) Tumorogenicity of NE-1.3 and LNCaP cells in athymic nude mice. An aliquot of 0.2 ml cell suspension that contained 1 $\times$ 10$^6$ NE-1.3 cells or 2 $\times$ 10$^6$ LNCaP parental cells were injected subcutaneously into mice (three mice per group). The tumor volume was measured weekly with calipers.
of NE markers. As shown in Fig. 5A, increased expression of RPTPα in LNCap cells resulted in elevated levels of multiple NE markers, including NSE, CgA, CgB, PTHrP, and NT, following a dose-dependent manner, despite the degree of CgA elevation being lower. In contrast, the Y789F mutant only had a marginal effect on the expression levels of those NE markers. The increased expression of RPTPα correlated with c-Src activation by increased phosphorylation at Tyr-418, while the Y789F mutant did not have a significant effect on c-Src activation (Fig. 5A). Furthermore, cells transfected with the wild-type RPTPα cDNA, but not vector alone (Fig. 5B) or Y789F mutant cDNA (data not shown), acquired a NE-like phenotype, such as dendritic-like or extended processes. The data collectively indicated that RPTPα plays a role in upregulating multi-NE markers and acquiring NE phenotype of LNCap cells.

We further examined whether c-Src mediates RPTPα signaling in the upregulation of NE markers in those cells. LNCap cells were transiently transfected with the wild-type RPTPα cDNA in the presence or absence of PP2, an inhibitor of c-Src family members. As shown in Fig. 5C, in RPTPα cDNA-transfected LNCap cells in the absence of PP2, c-Src was activated by increased phosphorylation at Tyr-418. In those transfected cells, ERK1/2 were also activated and NSE expression was increased. Importantly, PP2 effectively abolished RPTPα-induced Tyr-418 phosphorylation of c-Src and reduced ERK1/2 activation as well as NSE levels, following a dose-dependent manner (Fig. 5C). Additionally, 50 nM PP2 effectively blocked the induction of NE-like morphology in LNCap cells by an SR condition in a 5-day experimental period (data not shown). Again, the expression of Y789F RPTPα mutant had only a marginal effect on ERK1/2 activation or NSE expression level (Fig. 5C).

We determined the role of ERK1/2 in RPTPα-induced NE elevation by culturing RPTPα cDNA-transfected LNCap cells in the presence or absence of PD98059, the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor. As shown in Fig. 5D, in the absence of PD98059, increased RPTPα expression led to ERK1/2 activation as well as elevation of NSE, following a dose-dependent manner (Fig. 5D). These results collectively indicated that RPTPα signaling via c-Src and ERK1/2 plays a critical role in the transdifferentiation of androgen-sensitive LNCap cells into NE-like cells in the SR condition.

Since PP2 could effectively block the RPTPα signaling and SR condition-induced NE differentiation, we examined the effect of PP2 on the phenotype of NE cells. As shown in Fig. 5E, although PP2 effectively inhibited c-Src activity as indicated by decreased Tyr-418 phosphorylation, the morphology of those NE cells was not changed. The data further supported the notion that those NE subclone cells are terminally differentiated cells in which the regulatory mechanisms are irreversibly altered. Therefore, the phenotype of those cells cannot be reversed solely by
Figure 5 Effects of RPTPα signaling on the expression of NE markers in LNCaP cells. (A) LNCaP cells were seeded on 60 mm dishes and transfected with 2–4 μg cDNAs encoding the wild-type (wt) or Y789F mutant (mt) RPTPα protein. Cells that were transfected with the vector alone (vec) were used as a control. After a 2 day transfection, total cell lysates for each transfectant were separated by SDS/PAGE and transferred to nitrocellulose membranes for immunoblotting with antibodies against NSE, CgA, CgB, PTHrP, NT, phospho-Tyr-418 c-Src and c-Src protein, respectively. As a loading control, the level of β-actin on the same membrane was examined. Similar results were obtained in three sets of independent experiments. (B) LNCaP cells were plated on glass coverslips. After seeding for 48 h, cells were co-transfected with GFP expression vector plus the wild-type RPTPα cDNA (GFP-RPTPα) or vector alone (GFP-vector). After transfection, cells were maintained in the regular medium for 48 h and then fixed with 2% paraformaldehyde. The morphology of transfected cells with GFP was visualized by a fluorescence microscope at ×400 original magnification. (C) LNCaP cells were transfected with cDNA encoding, either the wild-type (wt) or the Y789F mutant (mt) of RPTPα protein, or the vector alone (vec) as a control. Twenty-four hours after transfection, cells were fed with the fresh medium in the
Effects of NE CM on LNCaP cell proliferation and PSA secretion

Clinical studies suggest that NE cells promote the proliferation of PCa cells during androgen-ablation therapy by secreting growth factors including PTHrP and NT (Abrahamsson 1999). We examined the effect of CM by NE cells on the proliferation of LNCaP cells in an SR environment. The CM from LNCaP cells was used as a control. As shown in Fig. 6A (upper left panel), the CM of NE-1.3 cells stimulated the growth of LNCaP cells in an SR condition. The growth stimulation initially followed in a dose-dependent manner with approximately 1.8-fold upregulation by 5% CM on day 7, while the growth stimulation by 10% CM was lower than that by 5% CM. A similar mitogenic activity was observed by the conditioned media from NE-1.8 cells on LNCaP cells (Fig. 6A, upper right panel). Nevertheless, the CM from LNCaP cells did not have a growth-stimulating effect (Fig. 6A, lower panel). To directly determine the paracrine effect by NE cells on LNCaP cell proliferation, the co-culture experiment was conducted. Fig. 6B clearly showed that the growth of LNCaP cells was promoted by the co-cultured NE-1.3 cells, but not by the co-cultured LNCaP cells or the medium alone.

The rebound of PSA in a patient’s circulation serves as a surrogate marker for the hormone-refractory growth of PCa (Sadar et al. 1999). We examined whether NE CM could increase PSA secretion by LNCaP cells in the absence of androgen. As shown in Fig. 6C, 5% CM by both NE cells increased the secreted level of PSA by LNCaP cells in an SR condition, but not the control medium. Furthermore, the NE CM-stimulated PSA secretion by LNCaP cells is not due to its effect on AR protein level (Fig. 6C). Together, these data clearly showed that in an SR environment, cancerous NE cells exhibit a paracrine effect on upregulating androgen-independent proliferation as well as PSA secretion by androgen-sensitive LNCaP cells.

Discussion

Despite the potential importance of NE cells in prostate carcinogenesis, the origin of the cancer-associated NE cells remains an enigma. The role of NE cells also requires further investigations. In this communication, we report the establishment and characterization of three NE subclone cell lines from LNCaP cells. These three cells acquire many features of NE cells seen in clinical prostate carcinomas, including irreversible NE phenotype, expression of multiple NE markers as well as partial epithelial markers, lack of AR or PSA expression and, importantly, slow growth rate with high tumorigenicity (Xue et al. 1997, Abrahamsson 1999, Ismail et al. 2002, Vashchenko & Abrahamsson 2005). To the best of our knowledge, this is the first study that clearly demonstrates the establishment of stable NE cell lines, which express multiple NE markers, transdifferentiated from PCa cells. The paracrine effect of these NE cells on the androgen-independent proliferation as well as PSA secretion by LNCaP parental cells in an SR condition is in parallel with clinical observations in the increased proliferation index of cancer cells adjacent to NE cells (Bonkhoff et al. 1995) and the phenomenon of rebound PSA level in patient’s circulation with hormone-refractory PCa (Chu & Lin 1998). Our data thus provide us with the crucial information in the origin of a subpopulation of cancerous NE cells and their role in prostate tumors, which greatly extends our current knowledge on prostatic NE differentiation.

Androgen-ablation therapy is commonly used for treating PCa patients, especially patients with metastatic PCa. It is well documented that clinical androgen-ablation therapy is associated with increased NE differentiation in prostate carcinomas (Jiborn et al. 1998, Ito et al. 2001, Ismail et al. 2002, Hirano et al. 2004). For example, Ito et al. (2001) reported that in archival specimens from 137 PCa patients, 70.5% of presence or absence of different concentrations of PP2 as indicated and maintained for an additional 48 h. Total cellular proteins were separated by SDS/PAGE. The phosphorylation levels of c-Src and ERK1/2 were analyzed by antibodies specific to phospho-Tyr-418 c-Src and phospho-ERK1/2, respectively. The protein levels of c-Src, ERK1/2, NSE, and β-actin were detected with corresponding antibodies. Similar results were obtained in two sets of independent experiments. (D) LNCaP cells were transfected with cDNA encoding the wild-type RPTPα protein, or the vector alone as a control. Twenty-four hours after transfection, the cells were fed with the fresh medium containing different concentrations of PD98059 (PD) as indicated and maintained for 48 h. Total lysates were prepared for Western blot analyses. (E) NE cells were seeded on T25 culture flasks. After 3-day incubation, cells were fed with the fresh medium in the presence or absence of 100 nM PP2. After 72 h incubation, cells were replenished with the corresponding fresh medium with or without PP2 and maintained for an additional 72 h. On day 9, the cell morphology was imaged and total cellular proteins were harvested for Western blot analyses.
Effect of CM by NE cells on the proliferation and PSA secretion of LNCaP cells. (A) $5 \times 10^4$ LNCaP cells were plated per well in six-well plates for 2 days. Cells were maintained in an SR medium for 2 days and then fed with the fresh medium containing different concentrations of CM by NE-1.3, NE-1.8, or LNCaP cells as day 0. Cells were counted on indicated times, and the remaining cells were fed with fresh corresponding medium on the same day. Each graph represents one set of experiments in triplicate. Similar results were obtained in three sets of independent experiments. (B) LNCaP cells were plated into porous inserts and maintained in the regular medium for 4 days. LNCaP and NE-1.3 cells were seeded into six-well plates and maintained in the corresponding medium for 3 days. The medium in inserts containing LNCaP cells was changed to an SR medium and those inserts were placed into wells containing LNCaP (LN/LN) or NE-1.3 cells (LN/NE) in each corresponding medium. As a control (C) inserts seeding LNCaP cells were placed in a well in the absence of cells with medium only. After 5 days incubation, the number of LNCaP cells in each insert in triplicates was counted. Similar results were observed in two sets of independent experiments. *$p < 0.05$ versus control or LN/LN. (C) $5 \times 10^5$ LNCaP cells were plated in T25 culture flasks in the regular medium for 2 days. Cells were then maintained in an SR medium for 2 days and fed with the fresh SR medium containing 5% CM by NE-1.3 or NE-1.8 cells. Cells fed with SR medium alone were designated controls. After a 3 day incubation, media and total cell lysates for each culture flask were collected and utilized for Western blot analyses to determine the secreted level of PSA and the expression level of AR, respectively. A Coomassie Blue-stained protein band on the nitrocellulose membrane and the level of $\beta$-actin were used as loading controls. Similar results were obtained in two sets of independent experiments.
68 patients who had received hormonal therapy for more than 13 months exhibited NE differentiation, compared with 44% of 25 patients who had received hormonal therapy of less than 12 months and 31.8% of 44 patients without therapy. Similar results were also shown in other studies (Ismail et al. 2002, Hirano et al. 2004). The data collectively suggest that androgen-ablation therapy enhances the NE differentiation of PCa cells. In this study, our NE subclone cells were established after LNCaP cells were cultured in androgen-depleted medium, mimicking the clinical phenomenon of the increased NE cell population after hormonal therapy. Further, androgen-ablation-induced NE differentiation is not a unique phenomenon to LNCaP cells, but also seen in androgen-sensitive human PCa MDA PCa2b cells with NSE elevation (data not shown) as well as the CWR22 xenograft tumor with elevated expression of synaptophysin, a NE marker (Huss et al. 2004). These results support the notion that, upon androgen-ablation treatment, androgen-sensitive PCa cells can transdifferentiate to become cancerous NE cells. These NE cells exhibit a highly aggressive phenotype as observed in clinics (Abrahamsson 1999) and thus are suitable for studying the molecular mechanism of NE differentiation in PCa cells during androgen-ablation therapy.

Interestingly, our data showed that LNCaP-SR and three NE cell types express keratin 8 and 18 (Fig. 2A), two prominent markers for luminal epithelial cells, and EpCAM, an epithelial adhesion molecule, more highly than LNCaP parental cells (data not shown). Similarly, in clinical archival specimens, over 90% of serotonin-positive prostatic NE cells (Xue et al. 1997) and two cell lines derived from prostatic small-cell carcinoma (van Bokhoven et al. 2003) express keratin 18. This is in parallel to the expression of PACP, a prostate epithelium-specific differentiation antigen, in all three NE cell lines (Fig. 2B and C) and clinical NE cells in prostate carcinomas (Abrahamsson 1999). Our data together thus support the notion that in prostate carcinomas, at least a subpopulation of cancerous NE cells are transdifferentiated from androgen-sensitive prostate cancerous epithelia during androgen-ablation therapy and thus those cells retain partial epithelial markers. Nevertheless, the NE cells in normal prostate do not express keratin (Vashchenko & Abrahamsson 2005), which implies that those NE cells may not be transdifferentiated from the keratin 8/18-positive luminal epithelial cells in the physiological condition. Thus cancerous NE cells express dual NE and epithelial markers, differing from normal NE cells.

The measurement of NE markers in the circulation of PCa patients is potentially one of the objective indicators of tumor progression, despite different results being obtained (Aprikian et al. 1993, Bubendorf et al. 1996, Abrahamsson et al. 1998). For example, initial studies revealed that circulating CgA level is elevated in the 48% of 25 patients with stage D2 PCa and is associated with its androgen-independent stage (Kadmon et al. 1991). Nevertheless, Schmid and colleagues (Schmid et al. 1994) reported that CgB is the major component, whereas CgA is virtually absent in 17 poorly differentiated prostate carcinomas. Similarly, a study of 22 PCa patients revealed that the serum level of CgB but not CgA was significantly increased during a 2-year period of follow-up (Angelsen et al. 1997). In cAMP-induced NE differentiation of LNCaP cells in culture, the CgA level was not altered (Bang et al. 1994). Similarly, all three NE cell types express a greatly elevated level of CgB, but only NE-1.9 cells express elevated CgA (Fig. 2A). One possible explanation for the discrepancy in observations is the heterogeneity of PCa cells that can be mediated by multiple pathways to NE differentiation, leading to more than one type of cancerous NE cell (Abrahamsson 1999, Zelivianski et al. 2001). Alternatively, CgA and CgB exhibit differential expression at different stages of cancer progression (Angelsen et al. 1997). Further experiments are required to clarify the discrepancies.

NT functions as a trophic factor for the growth of PC-3 cells (Seethalakshmi et al. 1997) and LNCaP cells in an SR condition (Sehgal et al. 1994). The high NT level in all three cancerous NE cell types (Fig. 2A) indicates that the NT signaling is downregulated by androgens, and in the SR condition NT may exhibit a paracrine effect. Similarly, PTHrP stimulates DNA synthesis in human PCa cells (Iwamura et al. 1995), and its expression in prostate carcinomas is higher than in BPH and normal prostate epithelia (Iwamura et al. 1995, Asadi et al. 1996). Interestingly, androgen-independent PC-3 and DU 145 cells express higher levels of PTHrP mRNA than androgen-sensitive LNCaP cells (Wu et al. 1998). The elevated levels of PTHrP protein in all three cancerous NE cells suggest a role of PTHrP in androgen-independent cell proliferation (Fig. 2A). Collectively, these three NE cell types express high levels of NE-stimulating growth factors as observed in clinical specimens. It is thus foreseeable that cancerous NE cells can promote the progression of PCa cells and its PSA secretion during androgen-ablation therapy, as seen in Fig. 6.

Studies in PCa archival tissues have demonstrated a close relationship between the expression levels of...
Bcl-2 and NSE (Segal et al. 1994). Similarly, the correlation of Bcl-2 expression with NE differentiation is also found in other cancers (Jiang et al. 1996, Atasoy et al. 2004). Our data show that LNCaP-SR cells express an elevated level of Bcl-2 compared with parental cells, lower than that in NE subclone cells (Fig. 2A). These results correlate with the observation that Bcl-2 expression in prostate tumors is upregulated by androgen ablation (McDonnell et al. 1992). These data collectively suggest a role for Bcl-2 in the survival of cancerous NE cells during androgen-ablation therapy.

In human prostate archival specimens, cancerous NE cells do not express AR. Although the short-term androgen-depleted LNCaP cells express AR (Zelivianski et al. 2001), the long-term androgen-depleted NE cells do not express detectable AR. The lack of AR and PSA expression in cancerous NE cell lines is consistent with that seen in clinical specimens (Sella et al. 2000). Significantly, even after long-term re-culturing in a growth condition containing the androgenic activity, those NE cells retain the NE phenotype and express NE markers, indicating a terminal transdifferentiation process. The data may also suggest that the suppression of AR expression is required for the terminal NE phenotype (Wright et al. 2003, Zhang et al. 2003). Thus, our NE cells resemble those cancerous NE cells in clinical samples (Xue et al. 1997, Ismail et al. 2002).

Multiple pathways of signal transduction are proposed to be involved in NE transdifferentiation from PCa cells (Zelivianski et al. 2001). This notion is further supported by the observation that vasoactive intestinal peptide (VIP) can induce the transdifferentiation of LNCaP cells to NE-like cells by activating protein kinase A, ERK and phosphoinositide 3-kinase (Juarranz et al. 2001, Gutierrez-Canas et al. 2005). In this study, our data suggest the involvement of RPTPα in this process, providing one of the mechanistic explanations for the clinical observations (Ito et al. 2001, Ismail et al. 2002, Hirano et al. 2004). The expression level of RPTPα correlates with NE differentiation of androgen-sensitive LNCaP cells in an SR environment (Fig. 4; Zelivianski et al. 2001, Zhang et al. 2003). Similarly, NCI-660, a human prostate small-cell carcinoma cell line, and MDA PCa2b-SR cells express high levels of RPTPα as well as NSE (D Kondrikou, FF Lin and M-F Lin, unpublished observations). Importantly, increased expression of the wild-type RPTPα, but not the Y789F mutant, correlates with the elevated expression of multi-NE markers and the NE phenotype. It should be noted that, compared with the high level of NSE in NE subclone cells, the relatively small 3–5-fold increase in the NSE level by RPTPα cDNA transfection is at least in part due to the low transfection efficiency of LNCaP cells, which is only approximately 15% (Zelivianski et al. 1998). Furthermore, c-Src via MEK/ERK1/2 is responsible for mediating RPTPα signals in prostate epithelium–NE differentiation. As
such, PP2 effectively inhibited c-Src activation and blocked the induction of NE phenotype in LNCaP-SR cells in a 5-day experimental period (data not shown). Thus, the critical role of RPTPα in PCa NE differentiation is similar to that of RPTPα in normal neuronal differentiation (den Hertog et al. 1993), PTP20 in the neuronal differentiation of PC12 cells (Aoki et al. 1996), and PTPβ2 in erythroid differentiation (Kume et al. 1996). The minor effects of Y789F mutant on NE marker expression may be due to retention of PTP activity in the mutant protein, which may activate alternative signal pathways to upregulate those gene expressions. Together, our data suggest the role of RPTPα in prostatic NE differentiation and RPTPα may serve as a useful marker of prostatic NE differentiation.

In conclusion, as shown in Fig. 7, our data support the hypothesis that in the absence of androgen, androgen-sensitive PCa cells express an elevated level of RPTPα that activates c-Src leading to ERK1/2 activation, which subsequently upregulates specific transcriptional factors for inducing multi-marker gene expression and acquiring the NE phenotype, while retaining partial epithelial markers. These transdifferentiated cancerous NE cells can secrete growth-stimulatory factors to promote directly the proliferation of PCa cells as well as PSA secretion in an SR environment, as observed in hormone-refractory PCa. In summary, these three NE cell types represent cancerous NE cells which provide us with a useful cell model system to investigate further the molecular mechanism of NE transdifferentiation and identify new targets for treating patients with advanced PCa.

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