Identification of Putative Stem Cell Markers, CD133 and CXCR4, in hTERT–Immortalized Primary Nonmalignant and Malignant Tumor-Derived Human Prostate Epithelial Cell Lines and in Prostate Cancer Specimens

Jun Miki, Bungo Furusato, Hongzen Li, Yongpeng Gu, Hiroyuki Takahashi, Shin Egawa, Isabell A. Sesterhenn, David G. McLeod, Shiv Srivastava, and John S. Rhim

Abstract

Understanding normal and cancer stem cells may provide insight into the origin of and new therapeutics for prostate cancer. Normal and cancer stem cells in prostate have recently been identified with a CD44+/CD133+/CD02+/E12+/CK18+/p63/high/CD133+ phenotype. Stromal cell–derived factor-1 (SDF-1) and its receptor, CXCR4, have multiple essential functions, including homing of stem cells and metastasis of cancer cells. We show here that human telomerase reverse transcriptase (hTERT)-immortalized primary nonmalignant (RC-165N/hTERT) and malignant (RC-92a/hTERT) tumor-derived human prostate epithelial cell lines retain stem cell properties with a CD133+/CD44+/CD02+/E12+/p63/high/CD133+ phenotype. Higher CD133 expression was detected in the hTERT-immortalized cells than in primary prostate cells. These immortalized cells exhibited "prostaspheres" in nonadherent culture systems and also maintained higher CD133 expression. The CD133+ cells from these immortalized cell lines had high proliferative potential and were able to differentiate into AR+ phenotype. In three-dimensional culture, the CD133+ cells from RC-165N/hTERT cells produced branched structures, whereas the CD133+ cells from RC-92a/hTERT cells produced large irregular spheroids with less branched structures. SDF-1 induced, but anti-CXCR4 antibody inhibited, migration of CD133+ cells from RC-92a/hTERT cells, which coexpressed CXCR4. CXCR4/SDF-1 may sustain tumor chemotaxis in cancer stem cells. Furthermore, immunostaining of clinical prostate specimens showed that CD133 expression was detected in a subpopulation of prostate cancer cells and corresponded to the loss of AR. Expression of CXCR4 was also detected in CD133+ cancer cells. These novel in vitro models may offer useful tools for the study of the biological features and functional integration of normal and cancer stem cells in prostate. [Cancer Res 2007;67(7):3153-61]

Introduction

Prostate cancer is the most common male malignancy in the Western world (1). Localized disease can be cured by surgery and radiation therapy. In contrast, the therapy most widely used against advanced disease is androgen ablation, and initially, it almost always produces objective clinical responses. However, most patients eventually relapse with ablation-resistant prostate cancer and develop metastatic disease; currently, there is no treatment that will cure progressive hormone-refractile metastatic prostate cancer. The mechanisms of progression of prostate cancer have been extensively studied yet are poorly understood. To explain these mechanisms and the heterogeneity in prostate cancer, one of the concepts that has evolved is that cancer arises from the neoplastic transformation of normal stem cells (2). Based on the stem cell model, understanding the stage of cell differentiation in normal prostate epithelium is essential for the identification of cells, which are involved in prostate carcinogenesis and ablation-resistant prostate cancer.

Normal prostatic epithelial stem cells have been shown to exist in the basal component. It has been proposed that androgen-independent stem cells give rise to two types of cells, stem cells and androgen-independent transit-amplifying cells, which can differentiate into luminal cells (3, 4). A small population of basal cells with the CD44+/integrin αvβ3/high/CD133+ phenotype was identified as human prostatic stem cells (5); this finding may strongly reinforce the assumption about the existence of prostate epithelium stem cells. Recent evidence supports the cancer stem cell theory [i.e., that tumors arise from cells termed cancer stem cells or tumor-initiating cells that have the ability of self-renewal and are responsible for maintaining the tumor (6)]. Collins et al. (7) have recently identified prostate cancer stem cells using the normal prostate epithelial stem cell markers CD44+/integrin αvβ3/high/CD133+.

CD133 is the human homologue of mouse prominin-1, a five-transmembrane domain glycoprotein and a cell surface protein originally found on neuroepithelial stem cells in mice (8). CD133 has been used to identify normal and cancer stem cells from several different tissues, such as hematopoietic (9) or leukemia (10) cells, neural (11) or brain tumor cells (12), and renal epithelial (13) or kidney cancer (14) cells.

Cancer stem cells have been postulated to maintain tumor growth, but it is still unclear how the mechanisms of spreading in these cancer stem cells follow a distinct metastatic pattern. The stromal cell–derived factor-1 (SDF-1)/CXCR4 axis, critical for the trafficking/homing of hematopoietic stem cells (15), was expressed...
in various types of adult stem cells, such as neural (16), liver (17), and skeletal muscle satellite cells (18). More recent evidence shows that the SDF-1/CXCR4 axis plays an important role in the trafficking of leukemia (19) and in metastasis of several solid tumors, including breast (20) and prostate (21–25). It has been postulated that the metastasis of cancer stem cells and the trafficking of normal stem cells share similar mechanisms because CXCR4 is highly expressed in several tumors as well as in their original normal tissue/organ-specific stem cells (26).

Using primary prostate specimens in stem cell studies carries the advantage that the cells indeed represent the original features of the tissue. However, there are still some difficulties, including the limited access to biopsy materials, the need for exclusion of contamination by cancer or normal cells, the short life span of the cells, and the small population of the putative stem cells (<1%). To circumvent these obstacles, an appropriate in vitro model is essential. Some immortalized prostate epithelial cell lines have been used as a model for normal prostate epithelial progenitor or stem cell lines (27). Several prostate cancer cell lines were shown to contain cancer stem cells by side population approach (28) or the cell surface marker CD44 (29). However, almost all of the available human prostate cancer cell lines were isolated from metastatic lesions or xenograft tumors, thus leaving a void in reagents representing long-term human cell lines derived from primary localized adenocarcinoma of the prostate. We have developed several primary nonmalignant and malignant tumor-derived human prostate epithelial (HPE) cell lines using a retroviral vector encoding human telomerase reverse transcriptase (hTERT) and have shown that they may serve as useful model systems for the elucidation of malignant transformation (30).

The expression of telomerase is associated with stem cells. Telomerase is usually repressed in normal human somatic cells but is found only in the stem cell compartment of several adult tissues (31). Low telomerase activity in normal prostate primary cells (32) and telomerase activation in ~80% of cases of prostate cancer have been observed (33), suggesting that the presence of normal stem cells and the immortality conferred by telomerase play key roles in cancer development. The observation of parallels between cancer stem cells and normal stem cells indicates that cancer stem cells are derived from normal stem cells or progenitor cells (6). Telomere shortening and telomerase activity seem to have a critical role in initiating carcinogenesis in cancer stem cells (34). In this study, we have investigated the presence of the putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized nonmalignant and malignant tumor-derived HPE cell lines and in prostate cancer tissues.

Materials and Methods

Cell culture. Primary cultures derived by the explant outgrowth method and immortalized cells generated by using hTERT transduction have been described (30). The hTERT-immortalized primary human prostate tumor-derived cell line (RC-92a/hTERT) and hTERT-immortalized primary benign prostate tissue-derived cell line (RC-165N/hTERT) were grown and maintained in a keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract and recombinant epidermal growth factor (EGF; KGM; Life Technologies, Inc., Gaithersburg, MD; ref 30).

Telomerase assay. Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP) assay using the TRAPEZE Telomerase Detection kit (Chemicon International, Temecula, CA).

Soft agar colony formation assay. To examine the anchorage-independent growth, a cell suspension (10^4 cells/mL) in 2 mL of 0.3% Noble agar (Difco Laboratories, Inc., Detroit, MI) with KGM was overlaid into six-well plates containing a 0.5% agar base. All samples were plated in triplicate. Colonies with >0.2 mm in diameter were counted on day 21.

In vivo tumorigenicity assay. The tumorigenicity assay was carried out in severe combined immunodeficient (SCID) mice. Briefly, 10^7 viable cells in 0.2 mL of PBS were injected s.c. into the middorsal intracapsular region of adult SCID mice. The SCID mice were observed for 6 months for the appearance of tumor development. RC-92a/hTERT cells produced a xenograft tumor, and SCID/5083-6 cells were established from this tumor.

Western blot analysis. Western blot assays were done as described previously (30). Blots were probed with the following antibodies: mouse monoclonal anti–androgen receptor (AR)-specific monoclonal antibody (AR 441, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-CXCR4 (Abcam, Cambridge, MA). Goat anti-mouse or anti-rabbit IgG-horseradish peroxidase antibody (Santa Cruz Biotechnology) served as the secondary antibody.

Clonogenicity/colony-forming assay. To determine the colony-forming efficiency (CFE), after cell sorting, 1 × 10^4 viable cells were suspended onto a 10-cm dish. All samples were plated in triplicate. After 21 days, the cultures were fixed and stained and colonies that contained >32 cells were counted. The CFE was designated as the number of colonies divided by the total number of seeded cells.

Prostasphere culture. Single cells were plated at 1,000/mL on a plate coated with 0.5% agar to prevent cells from attaching to the plate. Cells were grown in K-SFM supplemented with B27, 10 ng/mL EGF, and 10 ng/mL basic fibroblast growth factor (all from Invitrogen, Carlsbad, CA). Cells grown under these conditions as nonadherent spherical clusters were named “prostaspheres.” Prostaspheres were collected after 7 to 10 days and dissociated with Accutase (Innovative Cell Technologies, Inc., San Diego, CA). The cells obtained from dissociation were sieved through a 40-μm filter and analyzed by flow cytometry.

Flow cytometry. Fluorescence-activated cell sorting (FACS) analysis was used for determination of differentiation marker expression and CD133+/CD44+ cell sorting. We used the following monoclonal antibodies: keratin 1, 5, 10, and 14 (clone 34/E12; DAKO Corp, Carpinteria, CA), keratin 18 (clone DC10; DAKO), PSA (clone ER-PR8; Lab Vision Corp., Fremont, CA), integrin β1 (CD29; clone b37B; Lab Vision), integrin α5 (CD49b; clone 12F1- H6; Becton Dickinson Pharmingen, San Diego, CA), AR (clone G122-25; Becton Dickinson Pharmingen), CD44 (clone 513; phycoerythrin (PE) labeled; Becton Dickinson Pharmingen), p63 (clone 4A4; Santa Cruz Biotechnology), CXCR4 (clone 12G5; Becton Dickinson Pharmingen), and CD133/1 [allophycocyanin (APC) labeled; Miltenyi Biotec, Inc., Auburn, CA). For staining of cytoplasmic and nuclear antigens (keratins, CXCR4, AR, and p63), FIX&PERM (Invitrogen) was used. Zenon Mouse IgG Labeling Kits (Molecular Probes, Eugene, OR) were used to label antibodies with Alexa Fluor APC or PE. We included the corresponding isotype control antibody in each staining condition. Samples were analyzed with BD LSR II flow cytometer (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). Data were analyzed by either FACS Diva (Becton Dickinson Immunocytochemistry Systems) or WinList (Verity Software House, Topsham, ME).

Cells were sorted by FACS Aria (Becton Dickinson Immunocytochemistry Systems) and sort gates were defined on a dot plot of CD44 (PE) and CD133 (APC). For CD133+/CD44+ or CD133+/CD44+ populations, only the 5% of brightest and dimmest stained cells were gated.

Induction of AR expression. RC-165N/hTERT and RC-92a/hTERT cells were cultured for 14 days in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1 mmol/L R1881, or in KGM as a control, and then analyzed by Western blot. CD44+/CD133+ cells were isolated from both cell lines and cultured in the same conditions as described above and subsequently analyzed by flow cytometry.

Three-dimensional culture. After sorting, CD133+ and CD133− cells (at a density of 10,000/mL) were seeded on 24-well plates coated with Matrigel (BD Biosciences, San Jose, CA) and cultured for 10 days in KGM containing 2% Matrigel and 1 mmol/L R1881 with a medium change every 3 days. For morphologic differentiation analysis, branched and spheroid structures were scored in three different experiments with cells derived from RC-165N/hTERT and RC-92a/hTERT.

Cancer Res 2007; 67: (7). April 1, 2007 3154 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on July 22, 2017. © 2007 American Association for Cancer Research.
Migration assay. Cell migration assays were done in 6.4-mm-diameter chambers with 8-µm pore filters (Becton Dickinson Labware, Franklin Lakes, NJ). RC-92a/hTERT cells were placed in the upper chamber (10^4 per well) in K-SFM without supplement, and the lower chamber was filled with 600 µL of K-SFM containing various concentrations of SDF-1 (0, 10, and 100 ng/mL; R&D Systems, Minneapolis, MN). For neutralization studies, anti-CXCR4 monoclonal antibody (4 µg/mL; R&D Systems) was added to the upper compartment. Cells were incubated for 18 h at 37°C, 5% CO2. Nonmigrated cells on the top were removed following fixation and staining. Cell numbers in the lower chamber were counted using a 20× microscope objective (×200). Mean cell numbers were calculated per three high-power fields.

Statistical analysis. Results of CFE and migration assays were analyzed by paired t test. A P value of <0.05 was regarded as statistically significant.

Immunohistochemistry. Sixteen radical prostatectomies with clinically significant prostate cancer were studied from patients referred to Jikei University Hospital (Tokyo, Japan) for surgery between 1999 and 2001. The specimens were fixed in formalin, paraffin embedded, and processed into regular slides. Slides were deparaffinized and dehydrated to 100% ethanol, blocked with 0.6% hydrogen peroxide in methanol for 20 min, washed in PBS, and then microwaved in 10 mmol/L citrate buffer (pH 6.0) for 20 min. Microwave slides were allowed to stand at room temperature for 25 min, washed with PBS, and blocked with 1% normal horse serum for 20 min. Slides were incubated with the following primary monoclonal antibodies: anti-AR (dilution, 1:5; Vector Laboratories, Burlingame, CA), anti-CD133 mouse monoclonal antibody (1:10; Miltenyi Biotec), and anti-CXCR4 mouse monoclonal antibody (1:200; R&D Systems). Slides were rinsed with PBS and incubated with biotinylated horse anti-mouse (Vector Laboratories), and slides were rinsed with PBS and incubated in avidin-biotin complex (Vector Laboratories). Vector VIP (purple) was used as a chromogen substrate (Vector Laboratories). The slides were counterstained with hematoxylin.

Results

RC-165N/hTERT and RC-92a/hTERT cell lines have contrasting malignancy characteristics but have a similar undifferentiated phenotype. We evaluated whether RC-165N/hTERT and RC-92a/hTERT cells not only contained the original prostate epithelium characteristics but also were endowed with functional features of malignancy. Two cell lines, RC-165N/hTERT cells derived from nonmalignant tissue and RC-92a/hTERT cells derived from malignant tissue in prostate glands, had already been analyzed to assess their malignancy (Table 1; ref. 30). Morphology, doubling time, soft agar colony formation, and tumorigenicity in vivo correlate with the malignant behavior of the tissues from which they were derived.

In addition, to evaluate the characteristics of immortalized prostate epithelial cells, the expression of differentiation markers was analyzed by flow cytometry. The majority of cells in the two cell lines exhibited high expression of the low molecular weight cytokeratin CK18 in combination with the high molecular weight cytokeratins 34E12 (Table 1). We also stained for CD44 (35), α2 integrin (CD49b), and β1 integrin (CD29), which had already been reported as basal or stem cell markers (36). Almost all of the primary cells and the immortalized cells expressed CD44, α2 integrin, and β1 integrin (Table 1). The luminal cell markers AR and PSA were very low or not detectable (Table 1). Original primary cells of these immortalized cell lines showed almost same phenotypic characterization (data not shown). These results indicated that primary cells and hTERT-immortalized cells are populated by "transit-amplifying cells" or "intermediate cells" based on the presence of differentiation markers, including cytokeratins. Other investigators have reported similar results using primary (37) and immortalized prostate epithelial cells (27, 38).

Table 1. Characteristics of hTERT-immortalized HPE cell lines

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RC-165N/hTERT</th>
<th>RC-92a/hTERT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial site of origin</td>
<td>Benign prostate</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>Morphology</td>
<td>Polygonal</td>
<td>Spindle</td>
</tr>
<tr>
<td>Doubling time (h)</td>
<td>35</td>
<td>29</td>
</tr>
<tr>
<td>Soft agar colony formation (%)</td>
<td>&lt;0.001</td>
<td>0.155</td>
</tr>
<tr>
<td>Tumorigenic in SCID mice</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Expression of differentiation marker*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Integrin α2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>34E12</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CK18</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>AR</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PSA</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* The expression of differentiation markers was analyzed by flow cytometry. Level of expression: +, low (<10%); ++, medium (10–80%); ++++, high (80–100%); -, not detectable.

hTERT-immortalized HPE cell lines exhibit high expression of CD133 and maintain an undifferentiated condition as nonadherent prostapheres. Because hTERT-immortalized HPE cell lines have an undifferentiated phenotype, we entertained the question whether these cell lines contain immortalized normal and cancer stem cells. In prostate, normal and cancer stem cells have been identified with a CD44+/integrin α2/FWH/CD133+ phenotype (5, 7). We examined the expression of the CD133 marker in nonmalignant and malignant hTERT-immortalized HPE cell lines by flow cytometry. Dual staining with CD44 and CD133 by flow cytometry analysis shows that a CD44+/CD133+ population was detected in <1% of normal and primary cancer cells (Fig. 1A). This result is consistent with previous reports (5, 7). CD133 expression was detected in ~80% of RC-165N/hTERT cells, ~5% of RC-92a/hTERT cells, and ~30% of SCID5083-6 cells. It is notable that telomerase-immortalized cell lines showed higher expression of CD133 (>100-fold in RC-165N/hTERT cells and >10-fold higher in RC-92a/hTERT cells) than in their original primary cells, and the xenograft tumor cell line (SCID5083-6) induced by RC-92a/hTERT cells maintained higher level of CD133+ cells than its parental (RC-92a/hTERT) cells (Fig. 1A).

To determine the relationship between telomerase activity and CD133 expression, and whether it was conserved across different passages, CD133 expression was analyzed at several passages and at the same plating density in RC-165N/hTERT and RC-92a/hTERT cells. The TRAP assay showed that a high level of telomerase activity was detected at passage 8, when the cells were supposed to have overcome replicative senescence, but was not detectable at passage 2 (Fig. 1B). Higher CD133 expression was also observed at passage 8, and a similar expression level was maintained at later passages (Fig. 1B). This phenomenon was also seen in another hTERT-immortalized nonmalignant tumor-derived RC-170/hTERT cell line (data not shown; ref. 30).

It has previously been shown in suspension culture that neural stem cells and mammary epithelial stem cells generate...
nonadherent spherical clusters of cells, termed neurospheres or mammospheres, composed of stem cells and progenitor cells. A normal human prostate-derived immortalized cell line, RWPE-1, could grow as free-floating clusters of cells called prostaspheres (41). Single-cell suspensions of RC-165N/hTERT and RC-92a/hTERT cells could also grow in a SFM as nonadherent prostate spheres similar to neurospheres or mammospheres (Fig. 1C). Flow cytometry analysis showed that prostate spheres contained much higher percentages of CD133+ cells than when grown in a monolayer (e.g., 99% of RC-165N/hTERT cells and 28% of RC-92a/hTERT cells; Fig. 1D). hTERT-immortalized HPE cell lines contained a high level of CD133+ populations and maintained an undifferentiated condition as nonadherent prostaspheres.

Characterization of CD133+ cells: a high proliferative potential of CD133+ cells in vitro. To evaluate the characteristics of CD133+/CD44+ cells, we examined the expression of additional differentiation markers using flow cytometry. All CD133+ cells expressed 34hE12, integrin α2, and integrin β1 but not p63, AR, and PSA. However, most of the CD133+ cells expressed the luminal cell marker CK18 (Fig. 1D). The phenotype of CD133+ cells derived from nonmalignant and malignant hTERT-immortalized cells was identical, and these findings are consistent with previous reports using primary cells (5, 7, 27), except for the expression pattern of CK18. Our results show the phenotype of CD133+ cells to be CD133+/CD44+/α2β1 integrin+/34hE12+/CK18+/p63+/AR+/PSA-.

To determine whether CD133+ cells possess the proliferative potential as characteristic of stem cells, we purified CD133+ and CD133− cells by FACS from hTERT-immortalized HPE cell lines and compared their clonogenicity in vitro. We isolated CD133+/CD44+ and CD133−/CD44+ cells from RC-165N/hTERT and RC-92a/hTERT cells. The purity of the CD133+ and CD133− cells was 80% to

**Figure 1.** CD133 expression in primary and hTERT-immortalized prostate epithelial cell lines. A, flow cytometry analysis of CD133/CD44 expression in primary cells (RC-165N and RC-92a cells), hTERT-immortalized cells (RC-165N/hTERT and RC-92a/hTERT cells), and xenograft tumor cells (SCID5083-6) from RC-92a/hTERT cells. B, telomerase activity was analyzed at passage 2 (before transfection of hTERT) and passage 8 (after transfection). Expression pattern of CD133 at passages 2, 8, 15, and 50. C, generation of a prostasphere from a single cell in RC-165N/hTERT cells (days 0, 3, 7, and 10). D, flow cytometry analysis of CD133 expression in prostaspheres in RC-165N/hTERT and RC-92a/hTERT cells. Gray-filled area, isotype control; dashed line, monolayer culture; bold line, nonadherent sphere culture.

**Figure 2.** Characterization of CD133+ cells; CD133+ cells have a high proliferative potential. A, the differentiated phenotype of isolated CD133+/CD44+ cells in RC-165N/hTERT cells (top) and in RC-92a/hTERT cells (bottom) by flow cytometric analysis. Cells were stained with the following antibodies: integrin α2, integrin β1, 34hE12, p63, CK18, AR, PSA (bold line), or isotype control (gray-filled area). B, CFE of CD133+/CD44+ (black columns) and CD133−/CD44− (white columns) cells in RC-165N/hTERT and RC-92a/hTERT cells. Columns, mean; bars, SD. *, P < 0.05, statistically significant differences from the CD133− cells.
98%, respectively, as shown by postsort flow cytometry analysis. Immediately after sorting, the cells were plated to determine the CFE and the colonies were scored after 14 to 21 days of culture. The CD133⁺ cells had a CFE of 16.6 ± 1.4%, but the CD133⁻ cells had a lower CFE, 4.95 ± 0.7% in RC-165N/hTERT cells (P < 0.05; Fig. 2B). Similar results with RC-92a/hTERT cells showed that the CD133⁺ cells had a CFE of 17.3 ± 1.32%, but the CD133⁻ cells had a lower CFE, 4.75 ± 1.25% (P < 0.05; Fig. 2B), likewise with SCID5083-6 cells (data not shown). This observation suggested that sorted CD133⁺ cells have a higher proliferative potential than CD133⁻ cells in both nonmalignant and malignant hTERT-immortalized HPE cells.

**CD133⁺ cells can be induced to undergo phenotypic and morphologic differentiation.** To evaluate whether CD133⁺ cells isolated from hTERT-immortalized HPE cell lines are able to differentiate, we analyzed two types of characteristics, phenotype and morphology, under differentiating conditions. We looked at AR, which is one of the most important terminal differentiated phenotypes, as normal prostate gland and prostate cancer cells are androgen dependent. We reported the low level of AR expression in RC-165N/hTERT and RC-92a/hTERT cells in serum-free conditions (30). In the present study, DMEM with serum and androgen-induced AR expression was confirmed by Western blotting (Fig. 3A). We also showed that CD133⁺/AR⁺ cells differentiated into AR⁺ phenotypes by flow cytometry (Fig. 3B).

It has been reported that culture of HPE cells in the presence of Matrigel allowed morphologic differentiation (42). We found that there are mainly two kinds of structures, branched and spheroid (Fig. 3C), when cultured on Matrigel as shown for mammary epithelial cells (43). CD133⁺ cells from nonmalignant RC-165N/hTERT cells produced several ductal lobuloalveolar structures; in contrast, CD133⁻ cells from RC-165N/hTERT cells mainly developed small spheroids or budding structures with less branching morphology (Fig. 3C and D). CD133⁺ cells from malignant RC-92a/hTERT cells produced several large irregular spheroids with less branched structures, whereas CD133⁻ cells from RC-92a/hTERT cells produced small spheroids with much less branched structures (Fig. 3C and D). CD133⁺ cells isolated from nonmalignant RC-165N/hTERT cells possess both morphologic and phenotypic differentiation potential, whereas CD133⁻ cells isolated from malignant RC-92a/hTERT cells lost most of their morphologic differentiation potential.

**CD133⁺ expressing CXCR4 can induce migration in vitro; CD133⁺/CXCR4⁺ cells are detected by immunohistochemistry in prostate cancer tissue.** The isolated CD133⁺ cells from immortalized HPE cell lines were further characterized by visualizing the expression of CXCR4, which is involved in homing/tracking of stem cells, using flow cytometry and Western blotting. CXCR4 expression was detected by flow cytometry both on the cell surface and as intracellular CXCR4 after permeabilization of the cell membrane. Thirty-one percent of CD133⁺ cells in RC-165N/hTERT cells and 42% in RC-92a/hTERT cells coexpressed CXCR4 (Fig. 4A). Western blot analysis confirmed the expression of CXCR4 in these cells, whereas DU145 was used as positive control (Fig. 4B).

Next, the chemotactic effect of SDF-1 on CD133⁺ cells from RC-92a/hTERT cells was analyzed using a migration assay. The presence of 100 ng/mL SDF-1 resulted in a significant increased cell counts in the lower chamber (Fig. 4C). This SDF-1-dependent chemotaxis was neutralized by treatment with anti-CXCR4 antibody (Fig. 4C). Furthermore, we detected the CD133⁺/CXCR4⁺ cancer cells in clinical human prostate cancer (Fig. 4D).

**Expression of CD133 in benign glands, high-grade prostatic intraepithelial neoplasia, and cancer of human prostate.** We did immunohistochemistry on 16 radical prostatectomy specimens to examine the level of CD133 protein expression in clinical
specimens. CD133 protein was found to be predominantly in epithelial cells but not in the stroma cells. Weak to moderate CD133 staining was present in a cytoplasmic location in epithelial cells in benign, prostatic intraepithelial neoplasia (PIN), and cancer cells. In benign glands, CD133 immunoreactivity was mainly observed in basal cells (Fig. 5B). In PIN, CD133 was predominantly present in secretory cells and was expressed in a variable manner among the individual cases (Fig. 5D). A similarly variable expression pattern was also observed in cancer cells (Fig. 5D and E). No trend was observed correlating expression of CD133 in cancer and clinicopathologic variables, including clinical stage, pathologic stage, and grade, in this small population set.

In addition, we also examined the relationship between expression of CD133 and that of the AR. AR has been reported to be present in the majority of prostate cancer cell nuclei, but immunohistochemical staining results showed that cancer cells also showed a heterogeneity of ARs, in contrast to homogeneous staining in normal secretory epithelium (44). For AR staining, we used serial sections of the same 16 cases that were stained with CD133. Generally, when the cancer cells showed positive cytoplasmic staining for CD133 (Fig. 5E), AR was not detected in the nucleus (Fig. 5F), whereas the area of CD133+ cancer cells (Fig. 5G) showed positive nuclear staining for AR (Fig. 5H). To our knowledge, this is the first documentation that CD133 expression was observed in a subpopulation of prostate cancer cells and corresponded to the loss of AR (Fig. 5E–H), which is consistent with our in vitro results.

Discussion

We have shown here that cells expressing CD133, a putative prostate stem cell marker, were successfully isolated and characterized from hTERT-immortalized nonmalignant and malignant tumor-derived HPE cell lines. In addition, we succeeded in detecting a small population of CD133+/AR- or CD133+/CXCR4+ phenotypes in clinical prostate cancer specimens. Although telomerase is thought to be activated in stem cells and cancer cells, cells derived from normal and cancerous prostate tissues undergo senescence after ~30 population doublings and rarely become immortal when derived from primary clinical specimens (45). To overcome this tendency toward senescence, we developed several nonmalignant and malignant HPE cell lines using hTERT immortalization (30). It has been described that hTERT overexpression maintains many characteristics of the corresponding parental cells (46). Our findings indicate that most of the differentiated phenotypes of primary cells are maintained in these hTERT-immortalized cell lines and tumorigenicity in vivo correlates with the malignant behavior of the cells from which they were derived. However, a higher expression level of CD133 in hTERT-immortalized cells (>10- to 100-fold) distinguished them from their primary cells. High expression of CD133 was observed right after the cells overcame replicative senescence and was maintained during long-term culture. There was not a significant difference in the level of CD133 expression between parent and subclone cells as determined by limiting dilution cloning (data not shown). These findings suggest that the increase in CD133 expression is not simply an artifact of long-term culture, subclones, or plating density, although ectopic hTERT expression may affect this alteration. A similar observation was reported in human cord blood cell lines immortalized by hTERT and human papillomavirus type 16 E6/E7 genes after CD34+ human hematopoietic stem
cells were isolated (47). Interestingly, the nonmalignant hTERT-immortalized cell line RC-165N/hTERT showed higher expression of CD133 than the malignant cell line RC-92a/hTERT, and the same phenomenon was seen in other hTERT-immortalized cell lines that we had established (data not shown). hTERT expression may have contributed in some different manner in nonmalignant and malignant prostate epithelial cells to explain the difference in CD133 expression. A possible explanation is that hTERT seems to be activated more selectively in a small population of the CD133+ cells. It may support the hypothesis that normal stem cells with the CD133+ phenotype could be target for immortalization from cancer stem cells initiated by telomerase activation.

The CD133+ cells in nonmalignant and malignant hTERT-immortalized HPE cell lines retained stem cell patterns of behavior, including high proliferative potential and differentiation ability. Parallels have been drawn between somatic stem cells and cancer stem cells in that both types of cells differentiate, although somatic stem cells differentiate in a highly regulated manner, whereas cancer cells differentiate in a poorly controlled manner. Our differentiation assay on Matrigel verified this contrast (i.e., that...
nonmalignant CD133+ cells retained differentiation potential but malignant CD133+ cells mostly failed to differentiate). In contrast to this morphologic differentiation, AR expression was induced in both nonmalignant and malignant tumor-derived CD133+ cells; in fact, AR expression is observed in most prostate cancer cells. The AR has been known to have a crucial role in the progression and treatment of prostate cancer. Androgen ablation therapy remains the primary therapy for patients with metastatic prostate cancer. However, the majority of men will eventually have a relapse to an ablation-resistant state. The mechanism of this progression still remains elusive. However, many evidences indicated that the AR plays a central role in this transition. It has been shown that disruption of the AR by a specific antibody or ribozyme inhibited proliferation in ablation-resistant prostate cancer cells in the absence of androgens (48). Increased AR expression (49) or the aberrant AR signaling (the AR acts as an oncogene; ref. 50) was also necessary in this progression. Based on cancer stem cell theory, cancer stem cells are biologically distinct from non–cancer stem cells in the overall population of cancer cells and are thought to be resistant to current therapy. In the prostate, cancer relapses because androgen ablation therapy may not be effective for AR+ cancer stem cells (2).

As has been reported previously, CD133+ cells from primary malignant tumors risk being contaminated with nonmalignant CD133+ cells because low-calcium serum-free defined medium selects against prostate cancer cells (27). In our study, this medium allowed not only nonmalignant but also malignant cells to grow. We found that SCID5083-6 cell lines derived from a xenograft tumor of RC-92a/hTERT cells contained high levels of CD133 expression and the distinction of morphologic differentiation on Matrigel between nonmalignant (RC-165N/hTERT) and malignant (RC-92a/hTERT or SCID5083-6) hTERT-immortalized HPE cell lines. These findings suggest that our CD133+ cells in primary malignant tumors were not derived from CD133+ normal stem cells and were able to grow in low-calcium serum-free defined medium.

How can cancer cells escape from the primary site and metastasize to other organs? After a relapse with ablation-resistant prostate cancer, bone and/or lymph node metastases are a major cause of morbidity and mortality. Recent evidence suggests that the SDF-1/CXCR4 axis is crucial for metastasis in prostate cancer (25). CXCR4, which is involved in hematopoietic stem cell homing, is highly expressed in several tumors as well as in their original normal tissue/organ-specific stem cells. It has been postulated that the metastasis of cancer stem cells and trafficking of normal stem cells share similar mechanisms (26); however, the relationship between CXCR4 and prostate normal/cancer stem cells is not understood. Previous immunohistochemical studies have shown that primary and metastatic prostate cancer cells expressed CXCR4 and CXCR4 induced a more aggressive phenotype in prostate cancer (24). The expression of CXCR4 was shown to be higher in several prostate cancer cell lines than in normal HPE cells (21, 22). Here, we report CXCR4 protein expression in CD133+ cells of both nonmalignant and malignant cell lines and the detection, in clinical cancer specimens, of the coexpression of CXCR4/CD133. The migration or invasion of the metastatic prostate cell lines PC3 or DU145 was enhanced by SDF-1 (21–23). We also showed that SDF-1 regulated the migration of malignant CD133+ cells, and these effects were inhibited by anti-CXCR4 antibody in vitro. Our findings suggest that the CXCR4 axis may be essential for the progression of prostate cancer cells and may be a potential therapeutic target in hormone-refractory metastatic prostate cancer.

There has been speculation that the difficulty in detecting cancer stem cells in clinical prostate tissues by immunohistochemical methods may be because only a very small population is present. To our knowledge, detection of CD133 by immunohistochemistry in human prostate cancer cells has not been reported, although its expression in normal prostate basal epithelial cells has been documented (5). In this study, we were able to detect, in clinical tissue, the existence of small populations of CD133+ cells that also lacked nuclear AR expression. The expression of CD133 was also observed not only in normal epithelial basal components but also in PIN, which is widely accepted as a premalignant lesion. Our in vitro and in vivo immunohistochemical results support that part of cancer stem cell theory that says that prostate cancer initially appears to consist of a mixture of androgen-independent and androgen-dependent cells and that CD133+ cells may be involved in carcinogenesis and its progression.

The results obtained here have shown that these hTERT-immortalized primary nonmalignant and malignant tumor-derived HPE cell lines retained stem cell properties, including CD133+. CXCR4/SDF-1 cell signaling pathway may serve as chemomigration of cancer stem cells. In addition, immunohistochemical analysis of clinical specimens showed the presence of CD133+/AR+ and CD133+/CXCR4+ cells in a subset of cancers. These novel in vitro models may offer useful tools for the study of the biological features and functional integration of normal and cancer stem cells in prostate.

Acknowledgments

Received 12/10/2006; revised 1/11/2007; accepted 1/29/2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the flow cytometrists Karen M. Wolcott and Kateryna Lund (Biomedical Instrumentation Center, Uniformed Service University, Bethesda, MD) and the histologist technician Denise Young (Department of Genitourinary, Armed Forces Institute of Pathology, Washington, DC) for contributing to our work.

References


Identification of Putative Stem Cell Markers, CD133 and CXCR4, in hTERT–Immortalized Primary Nonmalignant and Malignant Tumor-Derived Human Prostate Epithelial Cell Lines and in Prostate Cancer Specimens

Jun Miki, Bungo Furusato, Hongzhen Li, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/7/3153

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/7/3153.full#ref-list-1

Citing articles
This article has been cited by 26 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/7/3153.full#related-urls

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

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

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.