Lineage Relationship Between LNCaP and LNCaP-Derived Prostate Cancer Cell Lines

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BACKGROUND. LNCaP and its derivative cell lines, which include C4-2 (and the related C4-2B) and CL1, are used as models of prostate cancer. Unlike LNCaP, the other cell lines show features of progressed disease such as metastatic capability and hormone independence. Analyses were done to determine if C4-2 or CL1 cells were selected from pre-existent subpopulations in LNCaP.

METHODS. Prostate cancer cells were characterized by cluster designation (CD) phenotyping. Specific cell populations were sorted by flow cytometry. DNA array analysis was used to probe differential gene expression.

RESULTS. CD phenotyping showed that CL1 and C4-2 (and C4-2B) were very dissimilar, and C4-2 was more similar to LNCaP. One common difference between LNCaP and its derivatives was CD26, in which virtually all C4-2 or CL1 cells were $CD26^+$ but only ~10% of LNCaP cells were $CD26^+$. The $CD26^+$ subpopulation of LNCaP was isolated and cultured in vitro. After culture, a high percentage of the cells (descended from the sorted cells) were $CD26^+$, in contrast to those sorted by CD13 or CD44. The cultured CD13 and CD44 populations did not show a high percentage of $CD13^+$ and $CD44^+$ cells, respectively. CD13 and CD44 are markers, in addition to CD26, for CL1 but not for C4-2.

CONCLUSIONS. C4-2 arose probably from $CD26^+$ LNCaP cells, while CL1 arose de novo. *Prostate 60: 98–108, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **C4-2; CL1; LNCaP subpopulations; CD26**

INTRODUCTION

LNCaP is a prostate-specific antigen (PSA)-secreting, androgen receptor (AR)-positive cancer cell line established from a lymph node metastasis [1]. Its transcriptome has been extensively characterized (http://www.pedb.org), especially for the gene subset under androgen regulation [2]. Since LNCaP cells retain the response to androgen, they can be experimentally treated by hormone manipulation either in vivo (by growth in castrated animal hosts) or in vitro (by growth in androgen-depleted media) to generate variants with stable genotypic and phenotypic alterations. The variant cells show not only gain of androgen independence but also metastatic capability [3].

Abbreviations: BPH, benign prostatic hyperplasia; EST, expressed sequence tag; MMP-9, matrix metalloproteinase; PBS, phosphate buffered saline solution; RT-PCR, reverse transcriptase polymerase chain reaction.

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C4-2 and C4-2B are two LNCaP derivatives that resulted from ''selection'' or ''induction'' by certain cells (stromal cells of human bone but not lung, kidney, or NIH3T3 cells) in concert with perhaps host epigenetic factors when LNCaP and inducer cells were co-implanted in animals. Specifically, 10^6 LNCaP and 10^6 stromal cells were inoculated into male athymic mice. After 4 weeks in a castrated host, C4 was obtained. Further co-implantation of C4 and stromal cells in castrated hosts led to the C4-2 line. C4-2 exhibits androgen independent growth associated with skeletal metastasis, produces metastases when injected either subcutaneously or orthotopically in intact or castrated mice, and shows tropism for the skeletal environment to produce bone metastasis. It shares common marker chromosomes with the parental LNCaP [4,5]. A selected derivative of C4-2 from bone metastasis, C4-2B, demonstrates a faster growth rate and is osteoblastic. The osteotropism of C4-2 or C4-2B presumably results from the interaction between cancer cells and bone stromal cells. It is thought that cancer cells perturb normal bone remodeling via secretion of factors that stimulate bone resorption and bone production [6,7].

CL1, a fast growing, highly tumorigenic, and androgen independent derivative, was obtained from LNCaP cells grown in culture under androgen-free conditions [8]. CL1 shows aggressive growth, with metastasis to bone and other organs when implanted orthotopically in mice. It is characterized by increased expression of growth and pro-angiogenic factors, and decreased expression of PSA, AR, and tumor suppressor genes, and it retains marker chromosomes of LNCaP [9].

For cell-type analysis, we previously showed that expression of cluster designation (CD) cell surface molecules could be used to differentiate LNCaP and other prostate cancer cell lines [10], and to identify the component cell types of the prostate parenchyma [11]. This was accomplished by flow analysis and immunohistochemistry using a set of more than 150 commercially available, well-characterized CD antibodies. When used collectively, the multiple CD antibodies can differentiate not only prostate cancer cells from normal cells, but also several types of cancer cells that are postulated to be the basis of heterogeneity in tumor behavior (ref. [11], unpublished data). The differential CD expression between normal and cancer cells is not unexpected since CD expression is linked to physiological conditions. More relevant to our present study, CD profiles are unique for individual prostate cancer cell lines [10,12]. Furthermore, CD expression can be utilized as a means to isolate specific cell populations [13]. In this study, we attempted to uncover the lineage relationship between LNCaP and its derivative cell lines.

MATERIALS AND METHODS

Prostate Cancer Cell Lines and Xenografts

LNCaP, C4-2, and C4-2B were grown in serum-supplemented RPMI1640 media with 10^{-8} M dihydrotestosterone. CL1 was grown in charcoal stripped media. Growth of CL1 in serum-supplemented media did not alter significantly its (CD) expression profile [12]. LuCaP xenografts were implanted and passaged in immune-compromised mice as previously described [14]. Like LNCaP, LuCaP 35 was established from a lymph node metastasis while LuCaP 41 was from a primary tumor. To obtain androgen independent growth, the host animals were castrated. For LuCaP 35-AD (androgen dependent), a tumor was harvested 5 weeks after subcutaneous implantation (passage 50); for LuCaP 35-AI (androgen independent), the host animal was castrated 4 weeks after implantation, and the tumor harvested 4½ months later (passage 49). For LuCaP 41-AD, a tumor was harvested after nearly 4 months (passage 12), and for LuCaP 41-AI, a tumor was harvested after 8 weeks post-castration (passage 12). Another LuCaP 35-AD was included in the LuCaP 41 analysis for comparison.

CD Phenotyping of Cells

For CD antibody labeling, cultured cells were trypsinized and resuspended in 50-μl aliquots of 0.1% bovine serum albumin–Hanks' balanced salt solution (BSA–HBSS). Fluorescent dye [principally R-phycoerythrin (PE)]-conjugated CD antibodies (0.1 μg) were added for 15 min at room temperature. All monoclonal CD antibodies were obtained from BD-PharMingen (San Diego, CA). After labeling, the cells were resuspended in 0.35 ml 2% formalin–HBSS for flow analysis. Omission of the primary antibody, or use of an irrelevant isotype-specific fluorochromated antibody, was employed as a negative control to delineate the autofluorescent cell population. Events outside this population were scored as positive. For each antibody specificity, 5,000 events (cells) on average were recorded, and the percentage of fluorescent (i.e., labeled) cells was calculated and presented in a histogram format. In the case of unconjugated primary antibodies, a second 15-min incubation with PE-conjugated goat anti-mouse light chain (Vector Labs, Burlingame, CA) was performed. To corroborate CD expression detected by flow analysis, cytospins of the cells were prepared and stained with CD antibodies at a concentration of 8 ng/μl. Immunocytochemistry was also done on cells cultured in chambered slides to ensure that cell trypsinization did not affect the expression of CD molecules. In either method, the cells were fixed in cold acetone for 10 min. An indirect avidin-biotin-peroxidase method was used

for immunostaining. The secondary antibody used for chromogen detection was a biotinylated anti-mouse IgG (BA-2000, Vector Labs). The slides were developed in a solution of diaminobenzidine and counterstained with hematoxylin. For the analysis of xenografts, the harvested tumors were cut and digested with collagenase at 37°C overnight in RPMI1640 media supplemented with 5% serum [13]. The cell suspension was filtered and aspirated through a 23-gauge needle, and then labeled with antibodies. No attempt was made to remove any mouse cells as the antibodies used were specific for human antigens. In this study, the reactivities to CD10, CD13, CD26, and CD44 were sufficient to distinguish the different cell lines. CD10 (neutral endopeptidase), CD13 (aminopeptidase N), and CD26 (dipeptidylpeptidase IV) are cell surface enzymes that process bioactive peptide molecules; CD44 is a receptor for hyaluronan in the extracellular matrix. In the normal prostate, CD10, CD13, and CD26 are expressed by luminal secretory cells whereas CD44 is expressed by basal cells. In primary tumors, CD26 is expressed by a majority of the cancer cells whereas CD10 and CD13 are expressed by a minority of the cancer cells; and CD44, being a basal cell marker, is usually absent [11].

Cell Sorting by Flow Cytometry

After trypsinization, LNCaP cells were resuspended in 100–200 μl of 0.1% BSA–HBSS, and the appropriate antibody conjugate (CD10-PE, CD13-PE, CD26-PE, or CD44-PE) was added to a concentration of $\leq$8 ng/μl for $\sim 1.5 \times 10^6$ cells. Cells were incubated without antibody as a negative control. After a 15-min incubation at room temperature, 1 ml of 0.1% BSA–HBSS was added. The suspension was centrifuged and resuspended in 0.5 ml 0.1% BSA–HBSS for sorting by FACStar^{Plus} (Becton Dickinson, Mountain View, CA) into individual wells of a 24-well plate, each containing 1 ml media. Negative cells were ''sorted'' from the autofluorescent population. The $CD10^+$, $CD10^-$, $CD13^+$, $CD13^-$, $CD26^+$, $CD26^-$, $CD44^+$, and $CD44^-$ populations were then cultured under the same conditions. Once the cell number reached $0.5–1.0 \times 10^6$, the populations were reanalyzed by flow.

Gene Expression Analysis by DNA Microarrays

A 40,000-gene chip was used to probe gene expression of the cell lines. With the number of expressed human genes estimated at below 35,000 [15], there is a high probability that most of the human transcriptome is represented on this chip. For example, these CD molecules were among those detected in C4-2 cells: CD9, CD10, CD26, CD63, CD71, CD81, CD151, which were shown by flow analysis or immunocytochemistry to be present. Other genes detected include the prostate cancer-associated hepsin and α-methylacyl-CoA racemase, and those related to bone biology such as osteoglycin, osteoclast stimulating factor, bone morphogenetic protein 2, and MMP-9. The technology of DNA microarray analysis entailed generation of spottable material, chip printing, fluorescent labeling of probes, hybridization of probes to the array, scanning of the result, quantification, and data analysis. For this human array, 40,032 cDNA clones were obtained from the IMAGE Consortium (Invitrogen/Research Genetics, Carlsbad, CA) representing 35,013 UniGENE clusters (5,019 were redundant), and all were successfully amplified for spotting. Clones were spotted on polylysine-coated slides with a robotic spotter (GeneMachines, Omnigrid, San Carlos, CA). Fluorescent probes were generated by reverse transcriptase and fluorescent dye (Cy3 or Cy5)-tagged dUTP. RNA was prepared by cell lysis in STAT60 solution (Tel-Test ''B'', Friendswood, TX). Approximately 30–50 μg total RNA was used per experiment. Hybridization and washing were performed in an automatic slide processor. After washing in 0.1× SSC, the slides were scanned with a confocal laser scanner (Axon, Union City, CA). The experiments were done in quadruplicate (including switching of fluorochromes). Expression ratios for each spotted cDNA were calculated from the intensity difference. Data analysis in scoring spot intensity and background was done by two spot-finding/extraction applications: Dapple (ISB) and GenePix (Axon). Intensity data was integrated and recorded, and a script (VERA and SAM) had been added that allowed robust statistical error estimation [16]. For each gene, the likelihood that it was differentially expressed was evaluated by a statistical measure, λ. A set of control experiments, in which two samples of the same were labeled with different dyes, was used to determine a suitable λ threshold based on an acceptable false-positive rate. Values $\geq$25 are considered to be indicative of differential expression.

RESULTS

Differential CD26 Expression Between LNCaP and Its Aggressive Derivatives

CD phenotyping was carried out on C4-2 and C4-2B, and the result was compared to the published one of LNCaP. Figure 1 shows the histogram display for C4-2 (that of C4-2B was similar). The major difference between it and LNCaP was that nearly all C4-2 cells in a population were positive for CD26 compared to only ~10% of LNCaP. Many other CD markers used showed a similar pattern between C4-2 and LNCaP (low percentages for CD6, CD13, CD24, CD33, CD38, CD57, CD90, CD97; intermediate to high percentages

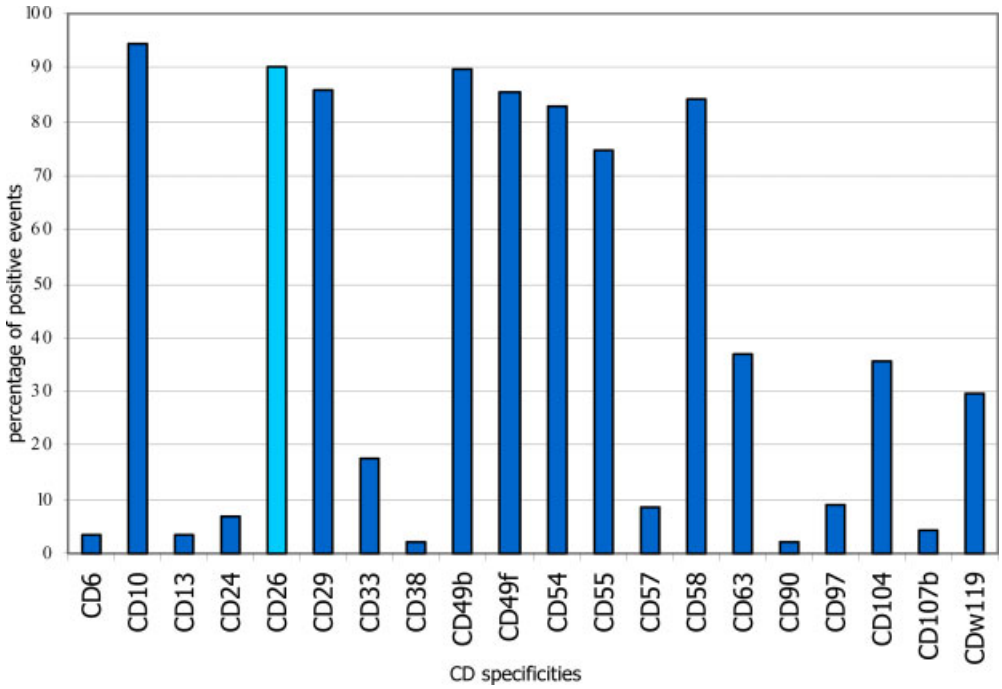


Fig. 1. Cluster designation (CD) expression of C4-2. The histogram displays the profile of C4-2 cells with regard to the expression of selected CD molecules identified on the X-axis. The percentage of positive cells is indicated on the Y-axis. In particular, the bulk of the population is positive for CD26 (fifth bar). C4-2 is also positive for CD10 (second bar). In this experiment, CD44 was not used, and a second experiment showed that C4-2 was not positive for CD44 (data not shown). The complete CD profile of LNCaP was previously published (ref. [10]). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

for CD49b, CD49f, CD63, CD104, CDw119). The CD107b percentage was lower in C4-2; a small increase in CD44 positivity (not shown) and increases in CD54, CD55 were seen. That both C4-2 and LNCaP were typed positive for CD10 was noteworthy because the expression of CD10 was reported to be under androgen regulation [17], and would, therefore, be expected to be down-regulated in C4-2. Previously, CD phenotyping between LNCaP and CL1 showed the latter to be also positive for CD26 [12]. CL1, however, showed many more different CD reactivities such as presence of CD13 and CD44, and absence of CD10 as indicated by the comparative flow analysis of LNCaP versus CL1 in Figure 2. The dominant cell type in LNCaP was characterized by the CD phenotype of $CD10^{+}/CD13^{-}/CD26^{-}/CD44^{-}$, whereas that in CL1 was characterized

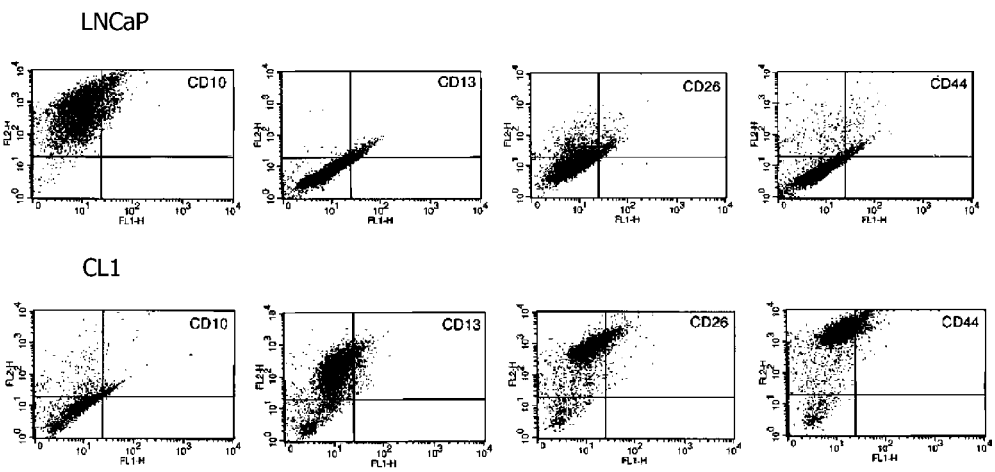


Fig. 2. Flow analysis of LNCaP and CL1. The first set of cytograms shows the reactivity of LNCaP cells and the second set shows the reactivity of CL1 cells. PE-conjugated antibodies to CD10, CD13, CD26, and CD44 were used for labeling. PE fluorescence is measured on the Y-axis (FITC fluorescence would be measured on the X-axis if a FITC-conjugated antibody were used). Percentages of positive cells were scored from 5,000 events collected. Not shown are the results of the no-antibody controls.

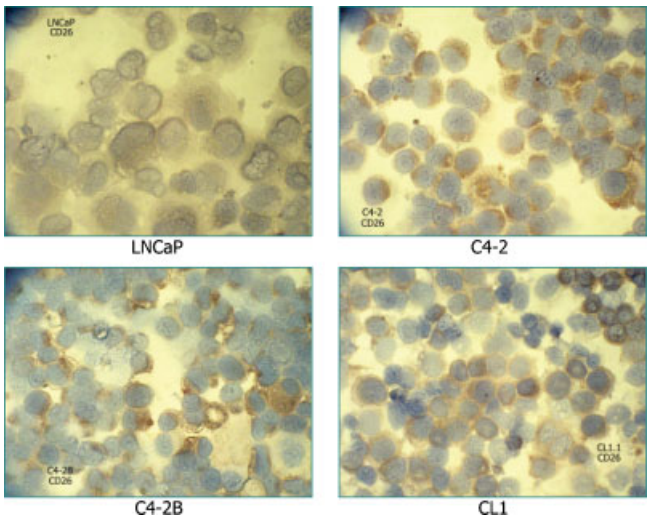


Fig. 3. CD26 expression in LNCaP and its derivatives. Cytospins were prepared for the different cell lines, and processed for CD26 immunocytochemistry. Shown are the results for LNCaP, C4-2, C4-2B, and CLI.I (a selected clone of CLI). Except for LNCaP, virtually all the cells in C4-2, C4-2B, and CLI are positive for CD26, as indicated by the brown stain.

by $CD10^-/CD13^+/CD26^+/CD44^+$. Note that the CD profile of CL1 closely resembles that of the PC3 prostate cancer cell line (cf. refs. [10,12]), but the morphological appearance of CL1 on culture dish is similar to that of LNCaP and readily distinguishable from that of PC3. The flow cytometry result of CD26 was evaluated by immunocytochemistry. Figure 3 shows cytospin preparations (in which cells were centrifuged onto slides) of LNCaP, C4-2, C4-2B, and CL1 stained by CD26. Nearly every cell in C4-2, C4-2B, and CL1 was positive for CD26 in contrast to LNCaP (virtually none of the cells in this field were stained).

Transcriptome Profiling for Differentially Expressed Genes

To determine whether genes other than *CD26* were differentially expressed between the two similarly CD-phenotyped LNCaP and C4-2/C4-2B cell lines, gene expression profiling using a 40,000-gene array was carried out. Total cellular RNA was prepared from the cell lines for labeling and hybridization. In agreement with the cell typing result, the expression of CD26 was found to be 11-fold higher in C4-2B compared to LNCaP as listed in Table I. Included in the table are 25 up-regulated genes in C4-2B with higher λ values (hence, statistically significant) than that of CD26. No other CD genes were detected to be significantly different to such a degree between the two cell lines. Nine genes showed a higher fold of change than that of CD26. The gene with the highest fold difference was a transcription factor, Krüppel-like factor 7 (*KLF7*), which could be involved in the alteration of gene expression in the derivation of C4-2. The top three genes overexpressed in LNCaP (not shown) were Y chromosome ubiquitin specific protease 9 (*USP9Y*), transcription factor ETS variant gene 1 (*ETV1*), and protein kinase inhibitor (*PKIB*). The expression of these genes might be relevant in prostate cancer progression.

LNCaP Subpopulations

Since the variant cell lines were derived from LNCaP, the question was whether these cells (C4-2- or CL1-like) were pre-existent in the LNCaP population. CD flow analysis of LNCaP population showed minor subpopulations that scored as $CD10^-$ (CL1-like), $CD13^+$ (CL1-like), $CD26^+$ (C4-2- and CL1-like), or $CD44^+$ (CL1-like). Cell binding to an array of several CD antibodies spotted on plastic also indicated that a given population of LNCaP cells contained cells not homogeneously labeled by various CD antibodies [10]. Accordingly, $CD10^-$, $CD13^+$, $CD26^+$, and $CD44^+$ cells were sorted individually from LNCaP (Fig. 4), and the sorted cells were allowed to expand in numbers by culture. For comparison, $CD10^+$, $CD13^-$, $CD26^-$, and $CD44^-$ LNCaP cells were also individually sorted and cultured. The following were sorted: 50,000 $CD10^+$ and 80 $CD10^-$ cells; 960 $CD13^+$ and 50,000 $CD13^-$ cells; 5,000 $CD26^+$ and 50,000 $CD26^-$cells; 4,500 $CD44^+$ and 50,000 $CD44^-$ cells. The $CD10^-$ well containing the fewest number of cells did not produce an outgrowth and hence was lost to the analysis (small numbers of LNCaP typically do not thrive). After 2 weeks, the $CD10^+$, $CD13^-$, $CD26^-$, and $CD44^-$ populations reached a sufficient level of cells for flow analysis. The cells were trypsinized and resuspended for labeling by CD10, CD13, CD26, and CD44 as was done for the unsorted LNCaP cells. Because of lower cell numbers to begin with, the other sorted populations took longer to expand. After 3 weeks, the $CD26^+$ and $CD44^+$ populations were ready for analysis; and after 5 weeks, the $CD13^+$ population was ready. Except for the $CD26^+$ sorted population, all other CD-sorted populations displayed a CD profile of these four CD specificities not remarkably different from that of non-sorted LNCaP (Fig. 5A,B). Remarkable was the high percentage ($\sim$80%) of $CD26^+$ cells seen only in the CD26 population versus $\sim$10% in all other populations. The culture of sorted $CD26^+$ cells was also serially passaged three-times, and the high percentage of $CD26^+$ cells was maintained in these passages (Fig. 5B,C). These results suggested that the $CD26^+$ LNCaP cells could represent a pre-existent or precursor population of C4-2 cells as both were typed $CD10^+/CD13^-/CD26^+/CD44^-$. However, other than CD26, this population

TABLE I. Differentially Expressed Genes Between LNCaP and C4-2B*

Gene name	Gene symbol	λ value	Ratio	Fold increase
Krüppel-like factor 7	KLF7	45.9	1.3071	20.28
Chromosome 1 open reading frame 24†	C1orf24	43.2	1.3755	23.74
Zinc α-2-glycoprotein 1†	AZGP1	42.9	1.1685	14.74
Acetyl-coenzyme A synthetase 2	ACAS2L	39.5	1.3371	21.73
Chromosome 1 open reading frame 24†	C1orf24	39.4	1.0103	10.24
Hypothetical protein DKFZp434F0318		36.9	0.922	8.36
Homo sapiens cDNA FLJ33790 fis		36.1	0.8232	6.66
Peptidylprolyl isomerase C	PPIC	34.5	0.6961	4.97
Cathepsin Z	CTSZ	34	1.1504	14.14
Hypothetical protein FLJ10462		33.6	0.7793	6.02
Chromosome condensation 1	CHC1	32.6	0.8078	6.42
Nicotinamide nucleotide transhydrogenase	NNT	32.4	1.2271	16.87
ESTs†		32.4	1.193	15.6
Zinc α-2-glycoprotein 1†	AZGP1	32.3	0.8111	6.47
KIAA1001 protein		32.3	0.8304	6.77
Hypothetical protein MGC39325		32.2	0.8683	7.38
Hypothetical protein FLJ32915		32.1	0.8993	7.93
BCL2-associated athanogene 2	BAG2	31.9	0.5972	3.96
EST		31.6	0.6727	4.71
ESTs†		31.6	1.3473	22.25
Collagen, type IX, α3	COL9A3	31.5	0.8894	7.75
GS3955 protein	GS3955	31.1	1.2856	19.3
S100 calcium binding protein P	S100P	31.1	0.5553	3.59
Up-regulated by BCG-CWS	LOC64116	31.1	0.5142	3.27
Epithelial protein up-regulated in carcinoma	DD96	31	0.9287	8.49
γ-Glutamyltransferase 1	GGT1	30.7	0.7773	5.99
Solute carrier family 2	SLC2A5	30.4	1.1116	12.93
Potassium channel, subfamily K, member 1	KCNK1	30	0.6239	4.21
Dipeptidylpeptidase IV (CD26)	DPP4	29.9	1.0365	10.88

*Genes whose λ values are larger than that of CD26 (indicated in **bold**) are listed. The ratio is the log intensity difference between Cy3 and Cy5 labeling of the individual array spots, and can be converted into x-fold difference in expression level. Three entries (marked by †) are repeated in this cohort: C1orf24, AZGP1, and one EST.

was unlike CL1 with regard to CD10, CD13, and CD44 ($CD10^+/CD13^-/CD44^-$ vs. $CD10^-/CD13^+/CD44^+$), and hence it could not represent a pre-existent population of CL1 cells.

CD Phenotypes of Xenografts

Since C4-2 and C4-2B were derived from interaction between LNCaP and stromal cells in vivo under androgen-free conditions while CL1 was not, we decided to analyze if androgen-free conditions (in castrated mice) without stromal cell interaction could select for altered clones with features (as defined by CD expression) of CL1. For this, we used LuCaP 35 and LuCaP 41, two xenografts developed in our laboratory. LuCaP 35 was like LNCaP in the CD pattern whereas LuCaP 41 was not. For comparison, the xenografts were grown and harvested from intact mice. LuCaP 35-AD (harvested from intact mice) showed a similar pattern of $CD10^+/CD13^-/CD26^-/CD44^-$ to that of LNCaP (data not shown). LuCaP 35-AI (harvested from castrated mice) showed increases in the percentages of CD13, CD26, and CD44 cells (Fig. 6, left), and these $CD13^+$, $CD26^+$, or $CD44^+$ cells could represent emerging CL1-like cancer cells. At the time of analysis, these cells did not appear to constitute the predominant population. Whether they will take over the population over time remains to be determined (which may not be possible as the animals are sacrificed within a prescribed period for humane reasons). Unfortunately, the LuCaP cells could not be cultured in vitro so that we could not sort the $CD13^+$, $CD26^+$, or $CD44^+$ LuCaP 35-AI cells to expand by cell culture as was done for LNCaP cells. The percentage of $CD10^+$ tumor cells (lower percentages of positive cells scored from xenograft tumors were due to ''contaminating'' mouse cells and particulate debris), however, did not differ between LuCaP 35-AD and LuCaP 35-AI (because CL1

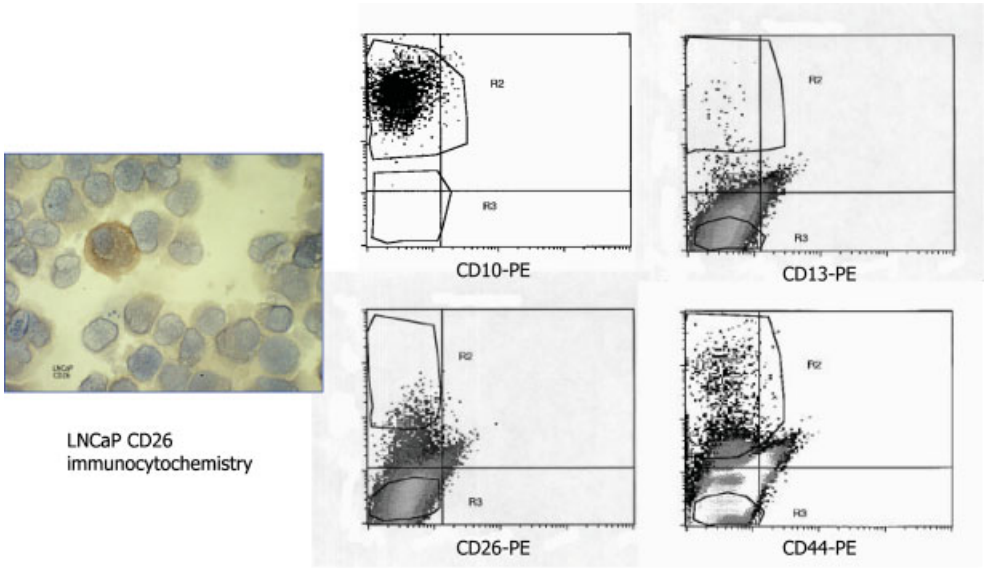


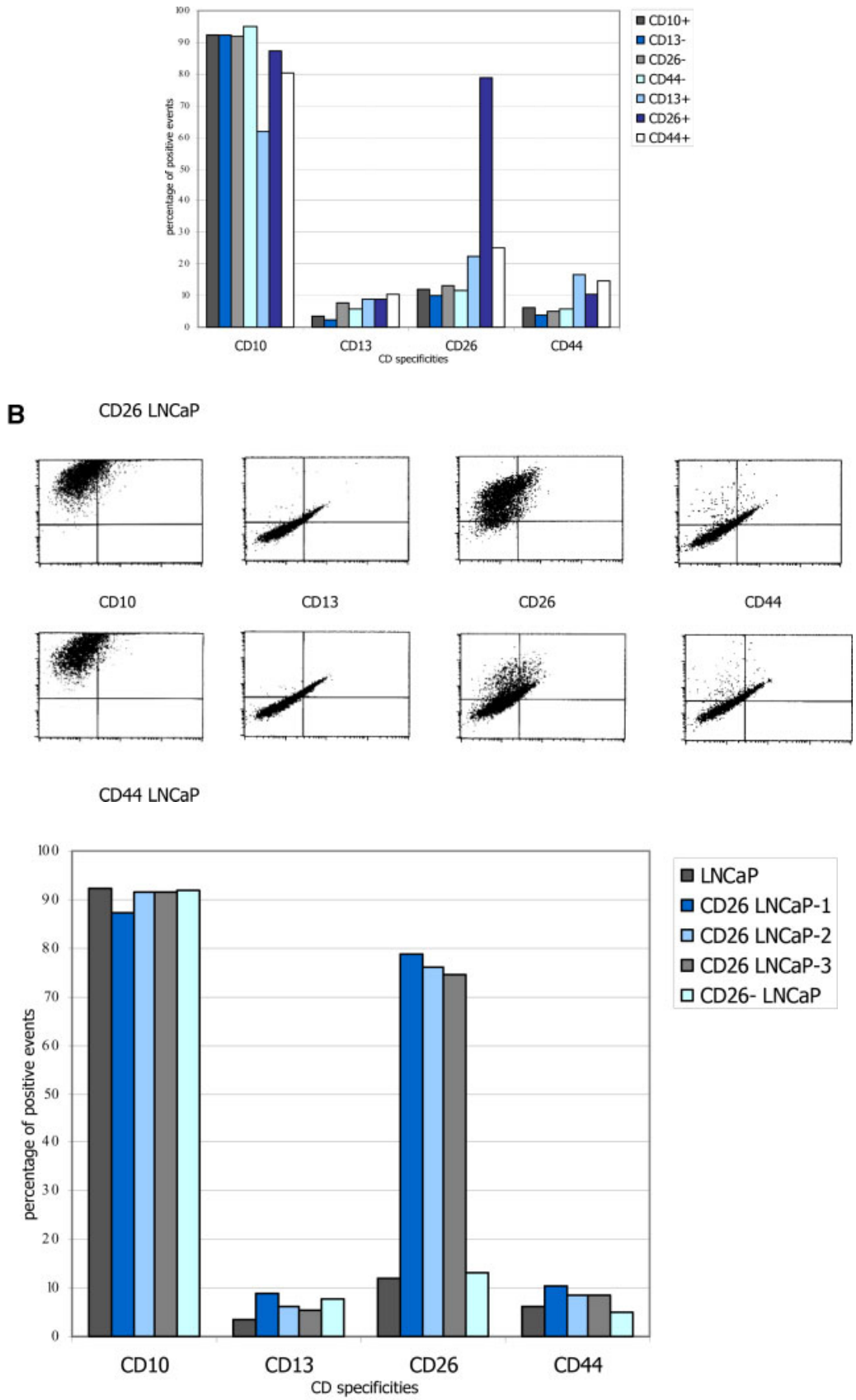
Fig. 4. LNCaP subpopulations. LNCaP cells were sorted into populations of $CDI0^+$, $CDI0^-$, $CDI3^+$, $CDI3^-$, $CD26^+$, $CD26^-$, $CD44^+$, and $CD44^-$ cells. The positive cells were sorted from the region outlined as R2, and the negative cells were sorted from the region outlined as R3 in the individual cytograms. The photomicrograph shows a cytospin of LNCaP cells stained with anti-CD26. A positive cell among many negative cells in this field is seen.

is $CD10^-$). Unlike LuCaP 35, LuCaP 41 contained a higher proportion of $CD26^+$ and a much smaller proportion of $CD10^+$ cells; and this was corroborated by RT-PCR analysis for CD10, CD13, CD26, and CD44 transcripts in the two tumors (Fig. 6, right and bottom). There were essentially no changes in the percentages of CD10, CD13, CD26, and CD44 cells between LuCaP 41-AD and LuCaP 41-AI. Androgen alone (vs. concurrent interaction with stromal cells) apparently did not affect dramatically the expression of these particular CD molecules in the xenograft population in vivo. None of the subcutaneously implanted xenografts showed detectable metastatic spread.

DISCUSSION

Gain of androgen independence and that of metastatic capability are both characteristics of progression in prostate cancer. Probably different sets of genes are responsible for the two cancer phenotypes. Some of these genes are likely those that encode CD molecules, perhaps more so with metastasis as that process entails cell–cell interaction mediated by cell surface molecules. One candidate in prostate cancer metastasis could be CD26. The C4-2 and CL1 cell lines are both metastatic and can proliferate without androgen yet their gene expression is very dissimilar. This is evident in their transcriptomes (ref. [12], unpublished gene expression data) and CD phenotypes. A common CD between CL1 and C4-2 is CD26. The differential expression of CD26 between the marginally tumorigenic, non-metastatic LNCaP (even though it was originally established from a metastasis) and its metastatic variants C4-2, C4-2B, and CL1 suggests that CD26 might be (one of several molecules) involved in cancer metastasis. CD26 is normally found in the luminal membrane of prostate epithelial cells [11,18]. CD26 activity is reported to be elevated in urological diseases, e.g., BPH [19] and prostate cancer [11,20]. There is quite a large amount of literature on the biological function of CD26. Of relevance is the role of CD26 in mediating cell migration and being responsible in part for the tissue-invasion phenotype. For example, CD26 expression appears in cells migrating

Fig. 5. CD analysis of sorted subpopulations. **A**: After in vitro culture, the resultant cells were re-analyzed for CDI0, CDI3, CD26, and CD44. The histogram displays the results for the $CDI0^+$, $CDI3^-$, $CD26^-$, $CD44^-$, $CDI3^+$, $CD26^+$, and $CD44^+$ populations (represented in different hues of the bars as indicated in the box inset). The notable result is the high percentage of $CD26^+$ cells in the sorted $CD26^+$ population (sixth bar in the CD26 specificity). A small increase in the percentage of $CD26^+$ cells is also seen in the sorted $CDI3^+$ and $CD44^+$ populations (fifth and seventh bar, respectively). **B**: Shown are the cytogram displays for the flow analysis of the sorted CD26 and CD44 populations. The increased percentage of $CD26^+$ cells is clearly evident in the CD26 LNCaP population. For each cytogram, the Y-axis is log PE fluorescence and the X-axis is log FITC fluorescence. **C**: The high percentage of $CD26^+$ cells in the CD26 LNCaP population is shown to be maintained in three serial passages (CD26 LNCaP-1, -2, and -3), compared to the sorted $CD26^-$ and unsorted LNCaP populations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


CD10+
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percentage of positive events
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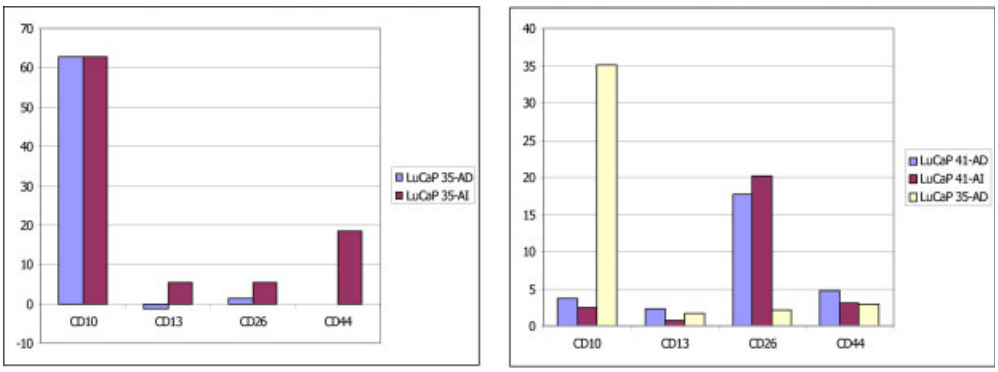
LNCaP
CD26 LNCaP-1
CD26 LNCaP-2
CD26 LNCaP-3
CD26- LNCaP
percentage of positive events
CD10
CD13
CD26
CD44
CD specificities

Fig. 5.



	CD10	CD13	CD26	CD44
LuCaP 35	++++++	–	+	+
LuCaP 41	+	+	++++	++

Fig. 6. CD expression of xenografts. The panel on the left contrasts the expression of CD10, CD13, CD26, and CD44 for an LNCaP-like xenograft, LuCaP 35, harvested from intact (AD) versus castrated (AI) hosts. The right panel contrasts the expression for LuCaP 41, which contained $CD26^+$ cells. Another sample of LuCaP 35-AD was analyzed in this experiment. Single cells were prepared from the tumors by collagenase digestion and CD expression was analyzed by flow cytometry. The xenograft RT-PCR results are included below the CD panels with the level of expression of markers tested indicated by the number of plus signs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

through connective tissue during wound closure. Specifically, an enzyme complex of CD26/separase is constituted at invadopodia of migratory fibroblasts [21]. CD26-expressing endothelial cells of the lung vasculature permit binding of tumor cells, whereas non-expressing endothelial cells in the vasculature of leg muscle, a nonmetastasized organ, do not [22]. This binding may be between CD26 and fibronectin/collagen. A published report showed that a CD26 fragment containing the fibronectin binding site could inhibit up to 80% lung colony formation by breast cancer cells [23]. CD26 also serves to bind plasminogen 2ε to initiate a Ca^{2+}-mediated signaling response that leads to an increase in the expression of MMP-9 [24]. MMP-9 enzymatic activity on the extracellular matrix is known to promote cancer cell migration. Many tumors, especially those of high-grade, are positive for MMP-9 expression, and so are lymph node metastases [25]. CD26 was shown to be useful in the rediagnosis of follicular thyroid carcinoma with distant metastasis. About 70% of the rediagnosed cases were positive for CD26, but only a very small percentage of adenoma and nodular hyperplasia were positive [26].

With regards to the genesis of the LNCaP derivatives, our results suggest that a possible lineage relationship between C4-2 cells, which are $CD26^+$, and $CD26^+$ LNCaP cells. Interaction between LNCaP and stromal cells may involve the selection of $CD26^+$ cells in the LNCaP population. Cell sorting showed that sorted $CD26^+$ LNCaP cells maintained their CD26 expression. Whether the $CD26^+$ LNCaP cells are metastasis capable or not remains to be tested. Xenograft LuCaP 35 cells prepared from tumors that resulted from subcutaneous injection had <10% CD26-staining cells as shown here, and no metastasis was observed [27]. More recently, metastatic LuCaP 35 variants were observed after orthotopic implantation [14,28], and these can be tested for CD26 expression. Our hypothesis is that the metastatic variant was selected through appropriate interaction with the mouse prostatic stromal cells (as provided via the orthotopic route) akin to the derivation of C4-2 from LNCaP and human bone stromal cells, and that the resultant variants would be positive for CD26 expression. Highly malignant LNCaP can also be promoted via the orthotopic route [29] (i.e., interaction with mouse prostate stromal cells leads to selection of the $CD26^+$ LNCaP cells within the tumor inoculum). Without stromal cell interaction, LNCaP cells may have to undergo many more genetic changes to become androgen independent and/or metastasis capable as represented in the derivation of CL1. Furthermore, adaptation to growth under androgen-free conditions alone may not be sufficient for the gain of metastatic capability, as demonstrated by the subcutaneously implanted LuCaP 35-AD/-AI xenografts, in which no metastasis was observed. For metastasis to occur, interaction with the appropriate stromal cells is required. Our results also suggest that CD expression has perhaps less to do with gain of hormone independence than with gain of metastasis.

The CD phenotype of LuCaP 41 ($CD10^-/CD26^+$, same as that of cancer cells in primary tumors) indicates that CD26 could not alone confer metastasis, since LuCaP 41 is not metastatic despite containing a sizeable proportion of $CD26^+$ cells. A metastasis co-promoting function may be contributed by other CD molecules

such as CD10 and CD13. Both, like CD26, are cell surface peptidases. Although cancer cells in both primary tumors and metastases are $CD26^+$, a majority of primary prostate tumors are negative for CD10 expression, whereas lymph node metastases almost invariably contain $CD10^+$ cancer cells (LNCaP and LuCaP 35 are both $CD10^+$). Indeed, CD10 expression correlates with liver metastasis of colorectal adenocarcinoma [30], and CD10 is upregulated in melanoma metastasis [31]. CD13, which is present in CL1 (negative for CD10), has been reported to confer an invasive phenotype on expressing cells [32]. Non-small cell lung cancer containing $CD13^+$ cells has a worse prognosis than that without [33]. Thus, these peptidases as a group could play a central role in prostate cancer metastasis. As these CD molecules are localized on the cell surface, they are prime therapeutic targets. We have generated an anti-CD26 (clone A6H) scFv in our previous studies [34,35], and a cytotoxic immunoconjugate can in principle be generated to target $CD26^+$ cancer cells in our mouse model.

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