Abstract

The expression of 119 cell surface molecules was catalogued for three prostate cancer cell lines, LNCaP, PC3, and DU145, all of which were established from metastases. Many of these molecules are common to all three cell lines, whereas some are differentially expressed. More prostate basal epithelial cell-specific than luminal epithelial cell-specific molecules are detected, especially in DU145 and PC3 cells. The cancer cells also express molecules that are not normally associated with prostate epithelial cells. As a population, expression of these molecules appears to be heterogeneous. This heterogeneity may be an inherent property of the population.

Introduction

In prostate cancer, tumor heterogeneity is a major problem in the clinical management of this disease. There is no reliable test to distinguish cancers that kill from those that may remain dormant. What makes one tumor more aggressive than another? One possibility is that tumors have different populations of cancer cell types. Some cell types are more malignant because of the particular gene products expressed by them (1). As a result, these cell types have metastatic capability or are resistant to hormone ablation. The cell type composition may not always be revealed by histology [the Gleason score (2)]. How can we then identify the more malignant types?

Normal prostatic glandular tissue is composed of the principal cell types luminal epithelial cells, basal epithelial cells, and stromal smooth muscle cells (3). Basal cells are the presumed progenitors of luminal cells (4, 5). The latter are the differentiated, postmitotic secretory cells of the prostate epithelium. Previously, I and others (6) presented a model of prostate cancer progression in which cancer cells arise from the transformation of luminal cells and can become more malignant by acquiring (progressively) the expression of markers specific to basal cells. As such, tumors can be comprised of more than one cancer cell type. Crucial to the identification of these cell types is the discovery of cell type-specific cell surface molecules that can be used in cell isolation by methods like flow cytometry. This allows one to study different cancer cell types individually with regard to their invasiveness, gene expression, and other biological properties. Furthermore, these molecules can, in theory, be targeted for therapeutic treatment of disseminated disease.

We reported recently that the cell surface molecules CD57 and CD44 can be used to identify two prostate cancer cell types. The CD44 type represents an advanced cancer cell type because it is found more frequently in primary tumors but more prominently in soft-tissue metastases (1). Normal luminal cells are positive for CD57, whereas basal cells are positive for CD44 (7). CD molecules are cluster of differentiation/designation antigens recognized by a set of monoclonal antibodies. These antibodies were originally raised against cells of the hematopoietic system. Thus, CD57 is identified as a natural killer cell antigen, and CD44 is identified as a lymphocyte homing receptor (8). CD molecules are attractive to tag cancer cells because their specific antibodies are widely available and because the functions of many of these molecules are relatively well known. However, CD57 and CD44 can only identify two prostate cancer cell types. There may also be subtypes of the CD57 and CD44 types. I propose to identify other candidate CD molecules by screening three commonly used prostate cancer cell lines, LNCaP, PC3, and DU145. These cell lines were all derived from metastatic lesions: LNCaP was derived from a lymph node; PC3 was derived from bone; and DU145 was derived from brain (9). Cell reactivity was detected by flow cytometry, a method capable of detecting a level as low as 1000 molecules/cell (10).

Materials and Methods

Cell Culture. The prostate cancer cell lines LNCaP (ATCC CRL 1740), PC3 (ATCC CRL 1435), and DU145 (ATCC HTB 81) were obtained from the American Type Culture Collection repository. Cells were cultured in the appropriate media recommended by the supplier supplemented with 10% FCS and glutamine. Near confluence, the cells were trypsinized and rinsed in media and HBSS. Viable cell count was done by using trypan blue exclusion.

Flow Cytometric Analysis and CD Antibodies. Aliquots of cultured cells were resuspended in 0.1% BSA-HBSS in a volume of 50 μl for antibody labeling and flow analysis. CD monoclonal (mouse IgG and IgM and rat IgG) antibodies conjugated to either R-phycocerythin (PE, 575 nm peak fluorescence) or FITC (530 nm peak fluorescence) were obtained from PharMingen (San Diego, CA), except for cCD49a-FITC, which was obtained from Endogen (Woburn, MA). Those specificities without an assigned CD number were T-cell receptor γδ, IL-8 receptor B, mannose receptor, NKB1, perforin, fusin, and integrin β3. About 0.05–0.1 μg of antibody was added to the cells, and the reaction was incubated in the dark for 15 min at room temperature. Isotype control was provided by irrelevant PE- or FITC-conjugated mouse MOPC21 (IgG1) and G155-178 (IgG2a) antibodies. The reaction was stopped by the addition of 1 ml of 0.1% BSA-HBSS. The cells were centrifuged and fixed in 0.35 ml of 2% paraformaldehyde. Flow analysis was done by a FACScan (Becton Dickinson, Mountain View, CA) machine fitted with a 488 nm laser. An isotype or no-antibody control was analyzed to delineate the unstained and autofluorescent population (7). Events that registered outside this trace were scored as positive, and 10,000 events were collected for each sample. The percentage of positive events was determined.

CD Antibody Array. CD reactivity detected by flow cytometry was checked by cell binding to the same antibodies immobilized on a plastic grid array. These arrays were constructed by spotting individual antibodies on the surface of Falcon 1034 polystyrene 65 × 15-mm Petri dishes with 10-mm grids. One dish could be spotted with 16 antibodies. Each spot was wet with 1 μl of PBS before 0.1 μg of antibody in 1 μl of PBS was applied. Antibody binding to the plastic surface was carried out at room temperature for 1 h. Afterward, the antibody array was rinsed in 0.1% BSA-PBS and incubated under a blocking solution of 1% BSA-PBS for 1 h. Cells were resuspended in small aliquots (<5 μl) of 0.1% BSA-PBS and added directly to the antibody spots. After a 15-min incubation at room temperature the array was washed well with 0.1% BSA-PBS. Cell binding was scored by light microscopy and photographed.

This method was also used to determine reactivity to fusin, integrin β3,
Fig. 1. CD profiles of prostate cancer cells. The percentages of positive events scored by flow cytometry are indicated on the Y axis, and the specificities tested are listed on the X axis for LNCaP (first panel), PC3 (second panel), and DU145 (third panel). The specificities are arranged in the order of CD1a, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CD11d, CD12, CD13, CD14, CD15, CD16, CDw17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32, CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41a, CD41b, CD42b, CD44, CD45, CD46, CD47, CD48, CD49a, CD49b, CD50, CD51/61, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CD60, CD61, CD62P, CD64, CD66b, CD69, CD71, CD74, CDw75, CDw78, CD79a, CD79b, CD80, CD81, CD82, CD85, CD86, CD87, CD89, CD90, CDw92, CD94, CD95, CD97, CD99, CD100, CD102, CD104, CD106, CD107a, CD107b, CD116, CD117, CD118, CD122, CDw123, CDw125, CD130, CD132, CD134, CDw137, CD147, CD152, CD154, T-cell receptor y8, IL-8 receptor B, mannose receptor, NKB1, and perforin. CD47, CD62L, CD62E, CD72, CD93, CD99R, CD114, CD121a, CDw125, CDw131, fusin, and integrin β7 are not included.
CD47, CD62L, CD62E, CD72, CD93, CD114, CD121a, CDw125, and CDw131 because these antibodies were not dye-conjugated.

**Gene Expression Analysis by RT-PCR**. RNA was prepared from LNCaP and PC3 cells and converted into cDNA. Gene sequences in the cDNA populations were detected by PCR and agarose gel electrophoresis of the reaction products as described previously (11). The primer pair sequences were as follows: (a) Ki67, GCAAATCATCCGAACCCGTGGTCATC and GGTGTTCAACTATTCTCAGTCCAGGAG; (b) CD10, GTGCCAGCAGTCCAACACTATTGAAC and CCCCCATTTCTGTGTTGGCAAGTC; (c) CD13, CATGTTTGACCGCTCCGAGGTCTATG and CACTGGAGCACCACCTCCTTGTTCTC; (d) CD26, TCCTCTACTATTAGATGTGTATGCAGG and GTATTTTGAGGTGCTAAGGTAAAGAGAAAC; (e) CD44, CAGATCGATTTGAATATACCTGCCGC and AGGGATTCTGTCTGTGCTGTCGGTGAT; and (f) CD81, GGGAGTGGAGGGCTGCACCAAGTGC and GATGCCACAGCAACACCATGCTC.

These sequences were selected so that: (a) they did not contain palindromes of 6 bp or longer; (b) their Tm would be 72°C, based on the empirical formula of 4°C per G/C pair and 2°C per A/T pair; and (c) their expected PCR products would be 400 bp in size.

**Single-cell Sorting and Culture**. PC3 cells were labeled with αCD44-PE in 0.1% BSA-PBS as described above. Single positive cells were sorted by FACStar Plus (Becton Dickinson) into individual wells of a 96-well plate. Cells from three regions of the cytogram representing low, intermediate, and high staining positivity were sorted. One hundred μl of RPMI 1640 media supplemented with 10% FCS were added to each well. After 3.5 weeks, positive wells were harvested for flow analysis. The PC3 cells in each well were trypsinized, rinsed with media, and resuspended in 40 μl of 0.1% BSA-HBSS for labeling with αCD44-PE. An aliquot was used for the no-antibody control.

**Results**

**CD Profiles of Prostate Cancer Cells.** A total of 119 CD specificities were screened for reactivity to LNCaP, PC3, and DU145. The results for 107 CD molecules, as determined by flow cytometry, are charted in Fig. 1. At a glance, these charts or CD profiles can serve as an identifier for each cell line. Although the cell lines were found to share many CD specificities, they could still be distinguished by unique ones. None of the cell lines was positive for the luminal cell marker CD57. PC3 and DU145 but not LNCaP were positive for the basal cell marker CD44. The reactivity could be grouped into four ranks, based on the percentage of positive events (>50%, 40–50%, 20–40%, and 10–20%) as seen in Table 1.

The common (>50% rank) CDs were CD9, CD29, CD49f, CD58, CD61, CD71, CD81, and CD147. CD44 was in the 20–40% rank for PC3 and DU145. Those specificities that scored below the 10% rank were considered negative. Reactivity to 12 other CD molecules was screened by a CD antibody array. All three cell lines were scored positive for CD47 and CD99R (data not shown).

Fig. 2 shows the level of expression for CD10, CD13, CD26, CD44, and CD81 in LNCaP and PC3 cells as analyzed by RT-PCR. Both cell types were positive for the cell proliferation-associated markers Ki67 and CD81. In agreement with the flow analysis result, PC3 was positive for CD13 but negative for CD10, whereas LNCaP was positive for CD10 but negative for CD13. PC3 was also positive for CD26 and CD44.

The positivity scored by flow cytometry was verified for some by the use of a CD antibody array. The method was a modified cell panning technique in which different antibodies were spotted on plastic in a grid to capture cells. Test cells were added over this grid, and bound cells were detected by light microscopy, as shown for LNCaP cells in Fig. 3. As expected, LNCaP cells were negative for CD44, CD26, CD49a, and CD57 and positive for CD81, CD107a,

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3 The abbreviations used are: RT-PCR, reverse transcription-PCR; IL, interleukin.
CD81, PC3 cells are positive for CD44, CD26, CD13, and cDNA. LNCaP cells are positive for CD10 and CD81; CD81. The blank lane is a PCR control without 760 bp for CD13, 610 bp for CD10, and 690 bp for Ki67, 600 bp for CD44, 690 bp for CD26, 860 bp for CD99, 660 bp for CD66b, and 690 bp for CD26. The PCR product sizes for the markers tested are 50% 40–50% 20–40% 10–20%. The results also showed that fewer cells were bound by CD10, CD95, and CD97 as compared with CD29. The CD10 and CD95 positivity detected by flow analysis was in the 20–40% rank, CD97 positivity was in the 10–20% rank, and CD29 positivity was in the >50% rank.

Population Heterogeneity. Because the labeling intensity was heterogeneous for the population, I wondered whether this heterogeneity was an inherent property of the cells. This issue was addressed by sorting single cells and culturing them into individual populations for reanalysis. Thus, PC3 cells were labeled with CD44-PE, and single cells were sorted from three fractions that scored low, intermediate, and high for CD44 staining. Single cells were cultured separately in a 96-well plate. About 15–20% of the wells were positive for growth, and the resultant populations (from low, intermediate, high CD44-positive cells) were analyzed by CD44 staining as shown in Fig. 4. All three populations displayed profiles similar to each other and to that of the presort population. Such an analysis was also done with LNCaP cells stained for CD10-PE, but single LNCaP cells could not be cultured (none of the wells was positive for growth).

Discussion

Expression screening of 119 CD molecules produced an informative CD profile for each cell line. The profiles of PC3 and DU145 are more similar than that of LNCaP, which is not surprising because it is generally thought that PC3 and DU145 are more alike in biological behavior and gene expression. Whereas LNCaP has retained the differentiated functions of prostate secretory cells in the synthesis of prostate-specific antigen and response to androgen regulation, PC3 and DU145 have not. In addition to the common CD molecules, PC3 and DU145 are positive for CD49b, CD54, CD55, CD97, and CD104.

CD71, CD29, and CD59. These specificities are detected in <50% of LNCaP cells. It would be interesting to sort the positive LNCaP cells and examine whether these molecules are coexpressed.

Because these cell lines were established from metastases, they therefore represent advanced disease cell types. Accordingly, none are positive for CD57, a luminal cell marker found in a majority of cancer cells in primary tumors and a marker of well-differentiated cancer (1). Other normal prostate epithelial cell markers that are not strongly positive (i.e., expressed in >50% cells) include CD24, CD38 (Ref. 12), CD64 (Fcγ receptor I), CDw75, CD82 (KAI1), CD117 (c-kit), and CDw123 (IL-3 receptor α). Normal epithelial markers retained by these cells are CD9, CD46 (membrane cofactor protein), CD47, CD99R, CD107a (lysosomal-associated protein LAMP-1), CD107b (LAMP-2), and CD117 (neurothelin). Because the cancer cells are epithelial in origin, they are negative for the prostate stromal cell markers CD49a (integrin α1), CD51/61 (integrin α5β1), and CDw131 (cytokine receptor subunit β). A detailed account of the reactivity of the CD antibodies to normal prostate cell types will be reported elsewhere.

PC3 and DU145 are positive for the basal cell markers CD49b (integrin α5), CD49f (α6), CD55 (decay-accelerating factor), CD59 (membrane-attack-complex-inhibitory factor), CD99R, CD104 (integrin βα1), and CD44. LNCaP is positive for CD49f, CD59, and CD99R only. The other markers are either negative (CD55 and CD44) or positive in some of the cells (CD49b, 40–50%; CD104, 20–40%). This finding is in line with a model of prostate cancer progression in which cancer cells acquire the expression of basal cell markers as the disease worsens (1, 6, 11). In this regard, PC3 and DU145 cells can be considered to represent more progressed cancer cell types than LNCaP. The α5β1 integrin complex of CD49b/CD29 present in PC3 and DU145 (and a smaller fraction of LNCaP cells) is reported to play a

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Table 1 Percentage ranks of CD positivities

Fig. 2. Differential CD gene expression. Expression analysis in LNCaP and PC3 cells was done by RT-PCR. HindIII is a DNA size marker (2.0 and 0.6 kb). The PCR product sizes for the markers tested are 860 bp for Ki67, 600 bp for CD44, 690 bp for CD26, 760 bp for CD13, 610 bp for CD10, and 690 bp for CD81. The blank lane is a PCR control without cDNA. LNCaP cells are positive for CD10 and CD81; PC3 cells are positive for CD44, CD26, CD13, and CD81.

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role in the interaction between prostate cancer cells and human bone marrow stroma (13, 14). Bone metastasis is a frequent outcome in prostate cancer. Bone metastases can be assayed for CD49b/CD29-positive cells, and the frequency of CD49b/CD29-positive cells in bone metastases can be compared with that seen in non-bone metastases. The expression pattern of integrin molecules by my flow analysis is compatible to that reported previously by other investigators (15, 16). Other basal cell markers such as CD90 (Thy-1) and CDw92 are not detected in any of the cell lines, whereas CD95 (Fas/APO-1 death receptor) is detected in the 20–40% fraction.

CD54 (intracellular adhesion molecule 1) and CD58 (lymphocyte function-associated antigen 3) are examples of non-prostate epithelial markers expressed by these cancer cells. In benign tissue, expression of CD54 is localized to vessel endothelial and nerve cells, and expression of CD58 is localized to the stroma. Another stromal cell-specific marker, CD56 (neural cell adhesion molecule), is not detected in these epithelial cancer cell types but is found to be expressed by a LuCaP (17) xenograft tumor with neuroendocrine differentiation as well as tumor cells in a liver metastasis of a deceased patient. CD97, which is found in PC3 and DU145 cells but not in LNCaP cells, is a heptaspanin transmembrane molecule whose expression may be associated with dedifferentiation because it is found in undifferentiated, anaplastic thyroid cancer but not in differentiated thyroid cancer or normal thyroid tissue (18). Normal prostate tissue and primary tumors show no reactivity to CD97. CD71 (transferrin receptor) and CD81 (target of an antiproliferative antibody 1) are characteristic of proliferating cells, although normal prostate cells also stain positive for these markers.

Fig. 3. Cell binding on CD array. The antibodies used in the assay are indicated on the individual panels. LNCaP cells were tested. Nearly all of the cells are positive for CD81, CD107a, CD71, and CD29. Smaller fractions are positive for CD39, CD10, and CD95. No cells were positive for CD44, CD57, or CD49a.

CD10 (neutral endopeptidase or common acute lymphoblastic leukemia antigen), CD13 (aminopeptidase N), and CD26 (dipeptidyl peptidase IV) are ectoenzymes that process bioactive peptides. All are luminal cell markers, and their expression (like that of the other luminal cell markers mentioned above) is diminished in cancer (19, 20). However, their expression pattern may be more complex. DU145 is negative for all three of these CD molecules, but PC3 is positive for CD13 and CD26, and LNCaP is positive for CD10. A LuCaP xenograft, such as LNCaP, established from a lymph node metastasis is also positive for CD10.5 CD13 appears to be down-regulated in CD57-positive cancer cells of primary tumors but is expressed by CD44-positive cancer cells present in soft-tissue metastases.5

Heterogeneity in expression may be an inherent population property of these cells. For example, PC3 cells can be sorted into single cells that stain high, intermediate, or low for CD44, and the sorted cells can be cultured separately. After the individual cultures have become confluent and are analyzed, a staining distribution similar to that of the original population is obtained. A thorough analysis would involve the examination of all other markers and isolation of sub-populations (e.g., CD44-positive versus CD44-negative PC3 or

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5 Unpublished data.
DU145 cells, CD10-positive versus CD10-negative LNCaP cells) to investigate their biological properties and gene expression.

It remains to be seen whether these three particular CD profiles can be detected in cancer cells of prostate tumors in patients. I think that the use of CD molecules to track prostate cancer cells can be applied to other types of adenocarcinoma to discover common themes.

Acknowledgments

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References

Differential Expression of Cell Surface Molecules in Prostate Cancer Cells

Alvin Y. Liu

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