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for Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) A prostate cancer cell's growth, differentiation and survival are guided by its interactions with the surrounding extracellular matrix (ECM). A number of promising therapeutic targets for androgen-independent and metastatic prostate cancers are contained within the signaling cascades downstream of the ECM-binding Integrin molecules. My research focuses on one component of these cascades, phospho-tyrosine kinase PYK-2, whose expression levels and activity I aim to manipulate in cell culture and within tumors in mice, using PYK-2 variant constructs and tetracycline-inducible promoters. PYK-2 manipulation, in cells of different metastatic potentials, may allow us to push cells forward or backward in cancerous progression, affecting cell proliferation, differentiation, migration and/or apoptosis. To date, I have: 1) characterized PYK-2 expression in prostate tissues and cell lines; 2) constructed tetracycline-regulated expression vectors of four PYK-2 variants, including constitutively-active and dominant-negative variants; 3) begun generating stable cell lines containing tetracycline-regulated PYK-2 variants; 4) linked GFP to PYK-2 and dominant-negative PYK-2 (PRNK), transiently transfected cells, and studied protein localization and construct effects on cell growth, apoptosis and migratory behaviors; and 5) quantified integrin subunit expression levels in each cell line, along with the tyrosine phosphorylation states of endogenous PYK-2, following cell adhesion to different extracellular matrix substrata.				
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Introduction

A prostate cancer cell's growth, differentiation and survival are guided by its interactions with the surrounding extracellular matrix (ECM). A number of promising therapeutic targets for androgen-independent and metastatic prostate cancers are contained within the signaling cascades downstream of the ECM-binding Integrin molecules. My research focuses on one component of these cascades, the phosphotyrosine kinase PYK-2, whose expression levels and activity I aim to manipulate in cell culture and within tumors in mice, using constitutively-active and dominant-negative PYK-2 constructs under the control of tetracycline-inducible promoters. Such regulation of PYK-2 may provide a means of pushing cells forward or backward in cancerous progression. By monitoring changes in the behaviors of cells expressing PYK-2 mutant variants, in the presence and absence of integrin-stimulating and blocking factors, we are simultaneously studying the downstream role of PYK-2 in regulating cell behaviors, and the upstream role of integrins in regulating PYK-2. Finally, we will introduce the PYK-2-construct-expressing cells into adult mice, that we treat with tetracycline at different times during tumor development, castrate or leave whole, and monitor for tumor progression and necrosis. An extremely valuable tool in these studies will be the lineage-related LNCaP cell lines, developed in our laboratory, each of which represents a stage in prostate cancer cell progression, from marginally-tumorigenic and androgen-sensitive, to highly-metastatic and androgen-insensitive. If intentional PYK-2 modulation proves sufficient to control LNCaP model cell proliferative, migratory or apoptotic behaviors, our laboratory is uniquely equipped to develop the approach into a clinical therapy, by delivering the genes in an adenoviral vector, first to LNCaP tumors in mice, and eventually to men.

Annual Summary Body

This research project focuses on Proline-rich Tyrosine Kinase (PYK-2), a key component of the signaling cascades downstream of the integrin cell-matrix adhesion molecules. We hypothesize that manipulation of PYK-2 activity and expression in prostate cancer cells will disrupt a link between the extracellular matrix and nucleus, allowing the intentional control of cell behaviors such as growth, migration, and apoptosis. This past year's work addresses the first of the three aims stated in our research proposal --we have constructed constitutively-active and dominant-negative PYK-2 variants under the control of a tetracycline-inducible promoters, begun stably transfecting cell lines with these constructs, and in the mean time have transiently-transfected cell lines with PYK-2 constructs. We have used these transiently-transfected cells for preliminary assays of PYK-2's regulation of cell migration, growth and apoptosis. We have also further characterized PYK-2 expression patterns in normal and cancerous prostate tissue and cell lines, along with its intracellular protein localization, using transfected PYK-2 labeled with green fluorescent protein (GFP). Finally, we have quantified integrin subunit expression levels in each of our cell lines, and have assayed the tyrosine phosphorylation states of endogenous PYK-2, following cell adhesion to different extracellular matrix substrata. In the coming year, after further assessing the effects of PYK-2 manipulation on cell behaviors in stably-transfected prostate cancer cell lines, we will link these effects upstream to Integrin $\alpha\beta3$ activity, by essentially repeating PYK-2 construct experiments, in the presence or absence of integrin-blocking or integrin-stimulating factors. Our third aim is then to evaluate, in adult mice, the androgen-dependence and metastatic potential of tumors containing PYK-2 construct-expressing prostate cells.

1) Characterization of PYK-2 expression patterns in prostate tissues and cell lines

The non-receptor tyrosine kinase PYK-2 was known to have a broad expression pattern, and to be particularly enriched in prostate tissues, brain, and hematopoietic cells (Avraham et al, 1995; Sasaki et al, 1995; Lev et al, 1995; Berg et al, 1997; Yu et al, 1996). We have further characterized PYK-2 expression patterns, strengthening the correlation between its elevated expression and cell assumption of greater metastatic potential, and providing further evidence of the LNCaP cell model's suitability for the proposed studies. We find PYK-2 expressed in normal prostate endothelia, muscle stroma and basal cells beneath columnar epithelium, but see elevated expression in these locations during Prostate Intra-epithelial Neoplasia (PIN) and in malignant carcinomas (FIG. 1). Likewise, using both Immunoprecipitation and densitometry techniques, we find PYK-2 levels to be two-fold higher in the androgen-independent, highly metastatic C4-2 and C4-2B cell lines than they are in the marginally-tumorigenic LNCaP cell line (FIG. 2). Thus, in addition to providing a means for comparing lineage-related cell types with progressively more advanced metastatic characters, our LNCaP cell model system reflects the expression patterns seen in patient tissues. Several other prostate cancer model cell lines do not appropriately express PYK-2 at any level, including DU145, PC3, and the antigen-immortalized epithelial cell line SV40T (FIG. 3).

2) Construction of tetracycline-regulated expression vectors of four PYK-2 variants , including constitutively-active and dominant-negative acting variants

Our ability to manipulate PYK-2 expression and activity is dependent upon the transfection of cell lines with constitutively-active and dominant-negative PYK-2 constructs under the control of a tetracycline-inducible promoter. We have now constructed four PYK-2 variants in tetracycline-inducible expression vectors, including one Wild-type PYK-2, two full-length, mutant PYK-2's, and a truncated PYK-2 (called PRNK) containing only the protein localization region. Our original constructs were made for use with a three vector tetracycline-regulated expression system, but because of difficulties in quickly

raising stably transfected cell lines with this system, we have now remade constructs for use with a new, two vector expression system (see below).

3) Generation of stable cell lines containing the tetracycline-regulated PYK-2 variants

For two reasons, we have yet to finish stably transfecting cell lines with constitutively active and dominant-negative PYK-2 constructs in tetracycline-inducible vectors. First, LNCaP model cell lines all divide slowly, on the order of one division every 2-3 days; to raise 3 million cells for screening from a single clone, therefore, takes 2-3 months. Additionally, the original vector system we used depends upon two separate transfection steps. The cells were first transfected with two vectors, grown to screening, transfected with a third vector, and grown to screening again. Six months after beginning the protocol, we screened over three hundred clones and could find only a few stably transfected cells. We have now retransfected with newly constructed vectors for use in a new, two vector tetracycline-regulated system, that requires only a single transfection step and growth to screening, and anticipate having stably transfected cell lines in 2 to 3 months. In the mean time, we have attached green fluorescent protein (GFP) to full length and truncated PYK-2 and have made transiently-transfected cell lines for use in preliminary studies

4) Protein localization, cell migration, cell growth, and apoptotic behaviors in cells overexpressing GFP-labeled PYK-2 and dominant-negative PYK-2 (PRNK)

While we have been working to establish stably-transfected prostate cancer cell lines expressing PYK-2 constructs under the control of tetracycline-regulated promoters, we have also transfected several cell lines transiently with green fluorescent protein (GFP)-labeled PYK-2 and dominant-negative PYK-2 (PRNK), and now have preliminary data on construct localization within the cells and construct effects on cell migration, cell growth, and apoptotic behaviors. Fusion proteins were expressed in androgen-dependent (LNCaP), androgen-independent (C4-2) and Control (p69; large T-antigen immortalized, human prostate epithelial) cells.

Protein Localization: PYK-2 protein localization is known to be cell line dependent; for example; PYK-2 is found concentrated in focal contacts within megakaryocytes (Li et al., 1996), but in a diffuse perinuclear pattern within rat fibroblast and smooth muscle cells (Matsuya et al., 1998, Zheng et al., 1998). PYK-2's localization was not known in the LNCaP model cell lines we are using, partly because of cross-reactivity between antibodies against PYK-2 and the similar protein Focal Adhesion Kinase (FAK). Our GFP-tagged PYK-2 construct revealed GFP-PYK-2, in all LNCaP model cell lines, to be distributed in a diffuse cytoplasmic pattern, with somewhat higher concentrations in the cell-cell borders of the p69 cells. We saw this same pattern in immunofluorescence studies using a new, more specific PYK-2 antibody from Transduction Laboratories (FIG. 4). Although LNCaP, C4-2 and p69 cells all lack distinct actin stress fibers and strong focal adhesions (as assayed with phalloidin and vinculin staining), they do all form lamellipodia and filipodia when attached to extracellular matrices, and it is interesting that GFP-PYK-2 showed even distribution relative to these structures.

Migratory Behaviors: We have assayed for shifts in cell migratory behaviors following over expression of our GFP-PYK-2 and GFP-PRNK constructs. Both PYK-2 and the closely-related Focal Adhesion Kinase (FAK) are implicated in the MAPK pathway, and as such, are likely regulators of cell motility. Overexpression of FAK has been correlated with increased metastatic and invasive behavior in a variety of cells (Sieg et al 1999, Gu et al 1999), however, migratory behaviors were notably reduced in our C4-2 cells

following over expression of either full-length or PRNK-truncated versions of the PYK-2 sequence (FIG. 5). The LNCaP cell line also was transiently transfected with PYK-2 constructs. No striking increase in motility was observed, and because untransfected LNCaP cells show very little migratory behavior, it was difficult to assay any decrease in motility. GFP-PYK-2 and GFP-PRNK transfected p69 cells are currently being assayed for migratory behavior. While our first assays have been haptotactic studies of cells moving toward a laminin substrate or within matrigel, we are currently assaying behaviors on vitronectin and osteopontin matrices.

Cell Growth and Apoptosis: Consistent with results in metastatic tumors, neither LNCaP nor C4-2 cells displayed anchorage-independent cell growth, as assayed by Fluorescence Activated Cell Sorting (FACS) analyses. And when cells from either cell line were detached from the substrate or deprived of serum, neither line displayed marked apoptosis, as assayed with TUNEL staining and FACS. To investigate PYK-2's involvement in cell proliferative and apoptotic decisions, we first followed cell growth responses to transient transfection of PYK-2 and PRNK, using MTT, and found that expression of either construct could decrease cell growth relative to controls, with PYK-2 expression resulting in a slightly larger decrease (FIG. 6). TUNEL staining after transient PYK-2 or PRNK transfection is underway, but preliminary data suggest that apoptosis is not increased in cells over expressing either construct. These studies will be repeated with stably-transfected cells expressing tetracycline-inducible constructs, allowing us to better manipulate expression levels, as these levels are likely to be important in PYK-2's interactions with co-factors.

5) Characterization of integrin subunit expression in prostate cancer cell lines, and assessment of PYK-2 tyrosine phosphorylation, following cell adhesion to different extracellular matrices

While activation of $\beta 3$ -containing integrins has previously been linked to PYK-2 phosphorylation (Duong et al. 1998; Duong et al. 1998; Damsky and Werb, 1992; Juliano and Haskill, 1993), and while we have found that elevated expression patterns of PYK-2 and $\alpha v \beta 3$ integrins are coincident, no comprehensive comparison had been made of integrin subunit expression patterns in prostate cell lines, nor had attachment-dependent PYK-2 phosphorylation been verified in our cell lines or comparisons made using different substrata. We have now used Fluorescence Activated Cell Sorting (FACS) analysis to determine the integrin subunit expression levels in a number of prostate cell lines, including LNCaP and C4-2 (FIG. 7). While very little change in integrin subunit *expression* was noted between cell lines, integrin subunit *usage* was markedly variable, with cells of different metastatic potential using different heterodimers to attach and spread on the same matrix (Edlund et al., submitted). We have also assayed the tyrosine phosphorylation state of endogenous PYK-2 in LNCaP and C4-2 cells, following their re-attachment to different extracellular matrices. We found that PYK-2 was phosphorylated in a time-dependent manner, as detected by immunoprecipitation and Western blotting, in both LNCaP and C4-2 cells, following attachment to both laminin and collagen IV. These studies address our second proposed aim of simultaneously assessing both upstream integrin regulation of PYK-2 and downstream PYK-2 regulation of cell behaviors.

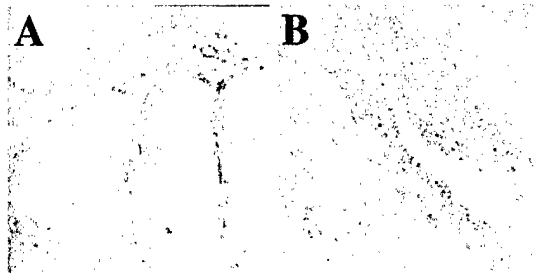


Figure 1. PYK-2 staining in tissue sections of human prostate (A) In normal tissue, PYK-2 is expressed at low levels in vessel endothelia, basal cells, and prostate stroma. (B) PYK-2 expression is elevated in hyperplasia.

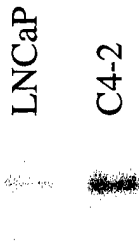


Figure 2. Western blot analyses of immunoprecipitated PYK-2 from LNCaP and C4-2 cells, revealing slightly-higher expression of PYK-2 in the androgen-independent cell line, C4-2.

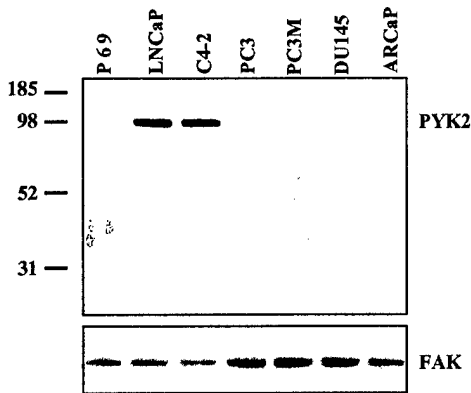


Figure 3. Western blot analyses of PYK-2 and FAK expression in whole cell lysates from LNCaP, C4-2, DU145, PC3, and SV40T prostate cell lines. Note that only LNCaP and C4-2 cell lines express PYK-2.

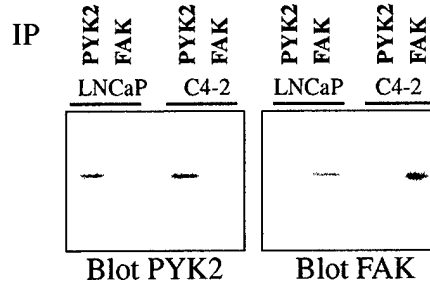


Figure 4. PYK-2 antibody specificity. FAK and PYK-2 were immunoprecipitated from whole cell lysates and stained with either FAK or PYK-2-specific antibodies, to check for cross-reactivity.

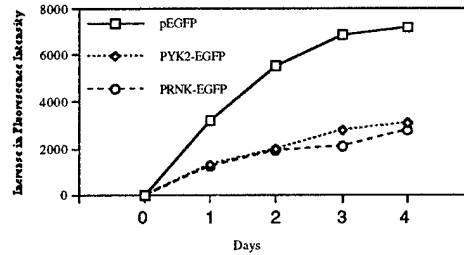


Figure 5. Cell migration in matrigel-coated Boyden chambers compared between mock-transfected and GFP-PYK-2 and GFP-PRNK transiently-transfected C4-2 cells. Invasion was assayed as an increase in fluorescence intensity in the lower chamber over time. Transfection with either PYK-2 construct reduced C4-2 migration.

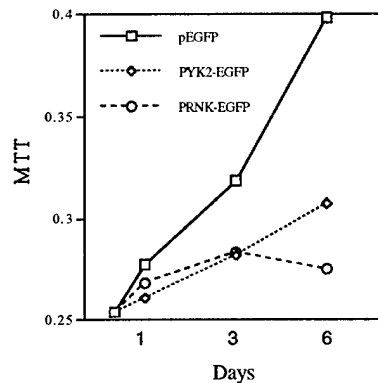


Figure 6. Growth inhibition of C4-2 cells following expression of either full-length PYK-2 or truncated PYK-2 (PRNK) constructs.

Expression of Integrins by FACS Analysis

	P69	LNCaP	C4	C4-2	C4-2B	ARCaP
α_1	3.1 (0.6)	1.7 (1.0)	1.2 (0.4)	1.8 (0.2)	1.1 (0.2)	28.2 (0.4)
α_2	183.6 (21.1)	7.7(1.2)	9.4 (0.4)	13.7 (0.4)	13.0 (3.2)	281.2 (44.4)
α_3	166.9 (5.6)	4.4 (0.6)	4.6 (3.2)	3.4 (1.2)	3.2 (0.0)	49.5 (8.2)
α_4	25.2 (7.6)	2.8 (0.0)	3.6 (0.7)	2.1 (0.2)	2.5 (1.8)	13.9 (0.6)
α_5	20.6 (6.0)	6.2 (0.4)	12.0 (2.4)	6.4 (0.4)	3.2 (0.0)	26.8 (2.8)
α_6	16.0 (4.4)	9.8 (0.4)	11.6 (2.2)	10.8 (0.8)	9.5 (0.2)	27.2 (0.6)
α_v	32.4 (6.4)	15.8 (0.8)	15.6 (0.4)	14.4 (1.4)	12.8 (.8)	22.6 (1.2)
α_{IIb}	1.2 (0.2)	1.3 (0.6)	1.2 (0.8)	1.4 (0.4)	1.1 (0.6)	1.1 (0.0)
β_1	39.8 (2.4)	17.2 (3.2)	17.7 (4.8)	15.5 (2.8)	13.6 (6.4)	97.2(10.0)
β_2	1.0 (0.0)	0.8 (0.4)	0.8 (0.0)	0.8 (0.0)	1.4 (0.6)	1.3 (0.2)
β_3	11.2 (0.4)	2.4 (0.8)	3.8 (0.4)	2.6 (0.4)	1.5 (0.0)	21.0 (0.4)
β_4	19.0 (3.2)	1.7 (0.6)	2.6 (0.0)	1.3 (0.2)	1.2 (0.8)	48.8 (4.8)
β_5	2.3 (0.2)	2.6 (0.2)	6.6 (0.5)	7.4 (2.7)	8.6 (3.4)	2.8 (0.3)
β_6	13.0 (1.6)	1.2 (0.4)	1.0 (0.0)	1.2 (0.4)	0.9 (0.0)	1.6 (0.8)
$\alpha_v\beta_3$		2.1 (0.4)		1.6 (0.4)		6.9 (0.4)

Figure 7. Integrin expression in LNCaP cells and their more metastatic, derived sublines C4, C4-2 C4-2B, as well as p69 cell line. Values for integrin expression are presented as the mean of two individual experiments with the range given in parentheses. An isotype non-specific antibody was used for control. All experimental fluorescence values are reported as the ratio of the control and specific fluorescence values.

Research Accomplishments

- Characterization of PYK-2 expression in prostate tissues and cell lines
- Construction of tetracycline-regulated expression vectors of four PYK-2 variants, including constitutively-active and dominant-negative variants
- Generating stable cell lines containing tetracycline-regulated PYK-2 variants
- Linkage of green fluorescent protein (GFP) to PYK-2 and dominant-negative PYK-2 (PRNK), transient transfection of LNCaP and C4-2 cells with these constructs, and assay of protein localization and construct effects on cell migration, growth and apoptosis
- Quantification of integrin subunit expression levels in several prostate cell lines, along with assay of PYK-2 tyrosine phosphorylation states, following cell adhesion to different extracellular matrix substrata.

Reportable Outcomes

Edlund, M., Miyamoto, T., Sikes, R.A., Ogle, R., Laurie, G.W., Farach-Carson, M.C., Otey, C.A., Zhou, H.E. and W.K. Chung (2000) Integrin expression and utilization by LNCaP prostate cancer cells on laminin substrata. (Submitted to *Cancer Research*).

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Integrin expression and utilization by LNCaP prostate cancer cells on laminin substrata

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Key words: Integrin, Laminin, LNCaP, Prostate cancer cells.

Abstract

The progression from benign to malignant prostate cancer requires migration of prostate cells through the basement membrane surrounding the epithelia and preferential cell relocation to the lymph nodes and bone. Past work on both normal and malignant prostate epithelial cells implicates the extracellular matrix proteins and associated soluble factors within the basal lamina and underlying stroma as important regulators of prostate epithelial cell behavior. To follow changes in prostate cell attachment to matrix proteins during disease progression, we have characterized integrin expression patterns in the parental LNCaP and its lineage-derived human prostate cancer sublines with progressively increasing androgen-independent, invasive, and metastatic potential.

Although the expression levels of the α_2 integrin subunit did differ slightly between the non-metastatic and metastatic cell lines (as seen by flow cytometric [FACS] analyses), the most striking shifts were found in integrin heterodimer *usage*, rather than its intrinsic expression level. In particular, the α_6 integrin subunit shifted its partner preference from β_4 in the parental androgen-dependent LNCaP cells to β_1 in the androgen-independent and more invasive C4, C4-2 and C4-2B sublines. The more invasive sublines also showed a similar increase in the use of α_V and β_3 integrin subunit combinations. Despite differences in integrin usage, both LNCaP and C4-2 cells attached to the matrix proteins laminin, vitronectin and osteopontin. However, only C4-2 cells migrated on osteopontin, a key matrix protein in the bone. Shifting integrin usage may account not only for adhesion differences between prostate tumor cells with vast differences in their androgen-dependency and metastatic potential, but also for cell responses to soluble prostate stromal components. Treatment with stromal cell conditioned media did not change the integrin expression profiles of the prostate cell lines, but interestingly, treatment did increase the spreading of C4-2, but not LNCaP, cells on laminin substrata. The increased spreading was

exquisitely sensitive to function-blocking antibodies against α_6 and β_1 , but not $\alpha_v\beta_3$ or α_2, α_3 and β_4 integrin subunits.

Introduction

Cancerous prostate cells are regulated in their differentiation, tumor growth and metastases by interactions with the surrounding cells and Extra-Cellular Matrix (ECM) in their microenvironments (1-3). Initial increases in the cells' invasive potential must be accompanied by decreases in cell-cell and cell-substrate attachment, as well as increases in cell motility. Such changes in cell behavior are believed to be potentiated by changes in and differential expression of adhesion receptors, including those adhesion receptors of the integrin family (2,3). Previous studies of prostate cancer have focused on the cell-surface distribution of integrins (4-8), as well as on correlating changes in integrin expression-level with changes in metastatic progression (9-10). The expression-level studies have taken two forms: first, cell lines with different metastatic potential, have been found to express different levels and subtypes of integrins, and second, within a given cell line, metastatic potential has been experimentally correlated with increases or decreases in levels of integrin expression (9,12,13).

Integrin molecular structure, and intra- and extracellular interactions with cytoplasmic regulatory proteins and ECM ligands provide tremendous potential for variation among cell types, well beyond that available through quantitative variation in expression level alone. Integrins are themselves heterodimeric molecules (consisting of one α and one β subunit), with at least 20 different combinations already described, many of which differ in extra- and intracellular binding specificities (14,15). "Inside-out" regulation of integrin heterodimer activity and subunit partner choices are thought to depend upon unique cytoplasmic regulatory protein repertoires, which differ among host cell types (16-18, 19 and references within). "Outside-in" regulation by integrins, in response to extracellular cues, has also been heavily studied and has revealed shifts in integrin gene expression, as well as changing integrin associations with numerous signaling molecules, including tyrosine kinases (FAK, pp60src), serine kinases (PKC, ERK, JNK and ILK), and lipid-intermediates (PI₃K and PI_{4,5}K; 15, 20, 21, 22 and references within). Hence,

integrin activity within a given cell is tightly coordinated with cell-cycle, behavior, and survival (14). Additionally, stromal components, like soluble growth factors and insoluble matrix proteins also modulate many integrin-dependent cell functions, including adhesion, migration and cytoskeletal organization (23, 24). Although a number of integrin variations during prostate cancer cell progression have been described, neither modulation of these variations by external factors, nor integrin heterodimer usage regulation during prostate cancer progression, are well understood.

The LNCaP-lineage cell model of prostate cancer progression (25-27) has given us the unique opportunity to follow integrin expression, usage, and cell behavior on a number of different extracellular substrata. LNCaP and LNCaP-derived cell lines are unique in that they vary in metastatic potential, but share a common genetic background. Previous phenotypic (27) and genotypic (28) characterizations of these cell lines revealed their remarkable resemblance to progressing clinical human prostate cancer. Here we seek to define cell behavioral parameters such as attachment, spreading and migration among lineage-derived cell lines at different points along the path to androgen-independence and invasive phenotypes.

We focus on characterizing interactions between cancerous prostate cells and their extracellular matrix microenvironments, particularly the ability of prostate cancer cells to attach, spread and migrate on laminin, a key protein in both the basement membrane surrounding the acini and in the tumors themselves (29). We also examine cell behaviors on several other matrix components found in bone (a favorite destination for prostate cancer cells following a metastatic cascade; 30 and references within). In comparison to benign tissues, integrin expression in bone tumors is sometimes altered, especially the expression of laminin-binding integrins (12, 13, 31, 32). One ECM receptor that binds laminin (among other components) is the heterodimeric integrin $\alpha_v\beta_3$, which is not expressed in normal prostate tissue, but is upregulated in prostatic adenocarcinoma (10). Likewise, in primary prostate carcinomas, shifts are seen in integrin

subunit α_6 's expression and heterodimerization partner choice, which appear to correlate with cancerous progression (8). In other cell types, the laminin-binding integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ have also been linked to invasive behaviors (6, 13). We used the LNCaP-lineaged human prostate cancer progression model to compare the laminin-binding integrin expression levels, heterodimer usage, and cell behaviors of cells with different metastatic potentials, but a common genetic background, as they attach and move on various matrix substrata.

Materials and Methods

Cell Culture, Antibodies and Extracellular Matrices

LNCaP cells and their more metastatic sublines, C4, C4-2, and C4-2B (26) were grown in T-media (Gibco, Rockville, MD) supplemented with 5 % fetal bovine serum. Primary cultures of prostate stromal cells were derived from the tissue surrounding prostatic adenocarcinomas, as described in Ozen et al., 1996. Conditioned media were prepared by adding fresh media when cells reached 80% confluency and removing it 48 hours later. Laminin-1 was purified from Engelbreth-Holme-Swarm (EHS) tumors according to Davis et al., 1989, based on the protocol of Kleinman et al., 1983. Osteopontin-2 was purified as described by Devoll et al., 1997. Vitronectin was purchased from Promega (Madison, WI). Antibodies to integrin subunits, α_2 , α_3 , α_6 , $\alpha_v\beta_3$, β_1 , β_3 and β_4 were all obtained from Chemicon (Emecula, CA). Vinculin antibody (V9131) was from Sigma (St. Louis, MO), and all secondary-conjugated antibodies were from Jackson Immunochemicals (West Grove, PA).

Immuofluorescent Confocal Microscopy.

Cells were seeded onto glass coverslips coated with 50 $\mu\text{g}/\text{ml}$ laminin-1. For immunocytochemistry, cells were allowed to spread, fixed in 3% formaldehyde, permeabilized in 0.2% Triton X-100, and stained, using either FITC-labeled phalloidin to label filamentous actin or anti-vinculin antibody (V9131) to detect focal adhesions. Texas Red-conjugated, goat anti-mouse secondary antibodies were from Jackson Immuno Research (Bar Harbor, ME). Cells were mounted on glass coverslips with gel-Mount (Biomedica Corp.), and images were acquired using a laser scan confocal microscope 410 (Carl Zeiss, Germany).

Flow Cytometry Analysis

Cells below 70% confluence were detached from tissue culture plates and suspended as single cells using a brief treatment of 10 mM EDTA, 20 mM HEPES buffer (pH 7.4) in T-media.

The EDTA was neutralized with CaCl_2 and MgSO_4 , and the cells washed again with T-media containing 0.1% BSA. 2.5×10^5 cells were used for each condition. Cells and primary antibodies (30 $\mu\text{g}/\text{ml}$) were incubated for 60 min. at 4°C, washed, and further incubated with secondary FITC-labeled goat anti-mouse (30 $\mu\text{g}/\text{ml}$) antibody for an additional 60 min., at 4°C. Following three brief washes, 1×10^4 cells were analyzed for fluorescence, using a FACS scan (Becton Dickinson, San Jose, CA). Cells treated with isotype-specific immunoglobulins served as controls. For both cell types, the relative fluorescence intensity was expressed as the increase over background fluorescence. Data points were presented as the mean of two independent experiments, with a range in parenthesis.

Substrate Adhesion, Attachment and Migration Assays

Cell attachment and competition assays were performed as in Vafa et al., 1998. Assay plates were precoated with laminin (from EHS tumors), vitronectin (Promega, Madison, WI), or osteopontin, by overnight incubation at 4°C, and were subsequently blocked with heat-inactivated BSA, for an additional 4 hours at room temperature. For adhesion assays, cells were trypsinized with 0.2% trypsin/ 2% EDTA in PBS pH 7.2, suspended in T- media for trituration to single cell suspension, and briefly centrifuged. Resuspended cells were then held in adhesion media (T-media with 20 mM HEPES pH 7.4, 7 mM EDTA and 0.1% BSA) for 5 hours at 37°C and 5% CO_2 , to ensure re-expression of integrins on the cell surface. After pre-incubation, CaCl_2 and MgSO_4 were added to neutralize EDTA. 5×10^3 cells, in 100 μl serum-free media, were added to each well and allowed to attach for 6 hour at 37°C. Triplicate cultures were prepared for each condition. After culture, cells were washed twice in PBS and stained using MTT (38, 39).

For the attachment assay with stromal-cell conditioned media, cell lines were grown to confluence, trypsinized, and replated (1:8) on tissue culture dishes, where they were allowed to grow for another two days, before being lifted and treated briefly with 10 mM EDTA, 20 mM HEPES buffer in T-media. After neutralizing the EDTA with CaCl_2 and MgSO_4 , the cells were

washed with T-media containing 0.1% BSA. Cells were finally held in conditioned media for 10 minutes, placed on laminin-coated dishes, allowed to adhere for 90 minutes, and then fixed in formaldehyde. The percentage of spread cells was scored for each cell line, and all values were normalized to control cells that had not been subjected to conditioned media.

Haptotaxis was assayed in triplicate using modified Boyden chambers, with an 8 μm pore size (Becton Dickinson, Bedford, MA or Corning, Acton, MA). 100 μl of PBS containing laminin (50 $\mu\text{g}/\text{ml}$), vitronectin (50 $\mu\text{g}/\text{ml}$) or osteopontin (20 $\mu\text{g}/\text{ml}$) was placed on the underside of the porous membrane and incubated at 4°C overnight. 100 μl of PBS alone served as a negative control. On the second day, chambers were assembled with serum-free T-media, containing 0.1 % BSA. 5×10^4 cells were added to the upper chambers and incubated at 37°C, 5% CO₂ of 16 hours. Cells were then fixed with 2% paraformaldehyde and stained with crystal violet. Cells remaining in the upper chamber were removed with a cotton swab. Cells that had migrated were counted using light microscopy; for each condition, 10 randomly-chosen fields of cells were counted, and the results presented as an average \pm Standard Deviation (SD).

Cell-surface Biotinylation

Integrins on cells surfaces were biotinylated, as previously described (40). Briefly, cells were washed in PBS and incubated with 500 $\mu\text{g}/\text{ml}$ Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 30 minutes, at room temperature. Cells were then washed in 50 mM glycine and PBS, before they were lysed (20 mM HEPES pH 7.4, 150 mM NaCl, 1 % NP-40, 2 mM PMSF 20 $\mu\text{g}/\text{ml}$ aprotinin and 20 $\mu\text{g}/\text{ml}$ leupeptin). Cell extracts were pre-cleared with protein A/G-agarose beads (Oncogen Science, Cambridge, MA) for 1 hour at 4°C, and spun at 10K rpm for 30 minutes. Integrin subunits were retrieved by immunoprecipitation: Integrin subunit-specific antibodies (200-500 $\mu\text{g}/\text{ml}$) were incubated with the cell lysate for 1 hour at 4°C, and immuno-complexes recovered using protein- A/G- coated agarose beads. Complexes were analyzed by non-denaturing 7.5 % polyacrylamide gel electrophoresis and electro-blotting. After transfer, filters

were blocked in 5% milk for 1 hour, at room temperature. Filters were then incubated with HRP-streptavidin and proteins detected using Enhanced Chemi-Luminescence (ECL) (Amersham, Piscataway, NJ).

Results

LNCaP parental and lineaged cell lines attach to laminin using different integrin subunits

Non-metastatic LNCaP human prostate cancer epithelial cells and their derivative metastatic sublines (C4, C4-2, and C4-2B) readily attached to a common laminin substrate, and all displayed numerous focal contacts and some poorly-developed stress fibers, as seen by staining for vinculin and filamentous actin. Representative vinculin and actin staining in attached LNCaP and C4-2 cells are shown in Figure 1; C4 and C4-2B cells stained similarly (data not shown).

To identify the integrins used for attachment by the different cell types, parental LNCaP and its derivative C4, C4-2 and C4-2B cell lines were selected (27) and specific, function-blocking integrin antibodies were added to attachment assays. Although the antibody staining suggested the formation of focal adhesion structures in all cell lines, the cells responded differently to the function blocking antibodies (Figure 2). Attachment of parental LNCaP cells was best blocked by antibodies against the subunits α_6 and β_4 , whereas, antibodies against these subunits did not effectively block attachment of C4, C4-2 or C4-2B cells, whose attachments, instead, were best blocked by antibodies against the intact $\alpha_v\beta_3$ integrin, and the subunits α_3 and β_1 . Attachment of all four cell lines was slightly reduced by antibodies against the subunit α_2 .

The differences in LNCaP and C4-2 cell attachment are not likely to be due to differential expression of integrin subunits

FACS analysis was used to determine the integrin subunit (α_2 , α_3 , α_v , β_1 , β_3 , β_4) expression levels in LNCaP and C4-2 cell lines (Table 1). A typical integrin flow cytometry profile is shown in Figure 3 for the integrin β subunits. Characterization of laminin-binding integrin levels by flow cytometry revealed only one difference in expression level (i.e. the α_2 subunit) among the four cell types. Although the expression of the integrin α_2 subunit in C4-2

cells was approximately double that in LNCaP cells, all other integrin receptors were found to remain constant in expression level across all cell lines (including C4 and C4-2B; data not shown).

Cell surface expression data was verified by immunoprecipitation of integrin subunits α_3 , α_6 , $\alpha_v\beta_3$, and β_1 from biotinylated cells of different cell lines (Figure 4).

Similar levels of the α_3 and β_1 subunit were precipitated in all cell lines. The α_3 subunit dimerizes most readily with the β_1 subunit, as seen by immunoprecipitation with either α_3 -specific or β_1 -specific antibodies (Fig. 4A). Although immunoprecipitation with an α_6 antibody co-precipitated β_1 and β_4 subunits from both LNCaP and C4 cells (Figure 4B), the $\alpha_6\beta_1$ heterodimer is not likely to be used for laminin attachment in LNCaP cells, as very little inhibition of cell attachment is seen by the β_1 antibody in LNCaP competition experiments (Figure 2). In comparison to LNCaP, very little β_4 subunit appears to be used for laminin attachment in the C4-2 and C4-2B sublines; the α_6 antibody did not immunoprecipitate as much of the β_4 subunit from these two cell lines (Figure 4B); and, a function-blocking antibody against β_4 did not inhibit their attachment to a laminin substrate (Figure 2), as it does for LNCaP. Although FACS analysis detected both α_v and β_3 subunits in all cell lines, at equivalent surface expression levels, immunoprecipitation with antibody to the $\alpha_v\beta_3$ heterodimer revealed nearly undetectable levels of $\alpha_v\beta_3$ in LNCaP cells, while readily detecting the heterodimer in all three derived sublines (Figure 4C). Use of the $\alpha_v\beta_3$ heterodimer does appear to be important for laminin attachment in the three metastatic sublines (but not LNCaP cells), as function-blocking antibody was able to inhibit cell attachment in the sublines (Figure 2).

The $\alpha_v\beta_3$ subunit is necessary for C4-2, but not LNCaP, cell attachment and migration

Because prostate cancer cells metastasize preferentially to bone, we were particularly interested in the integrin heterodimers known to interact with vitronectin (VN) and osteopontin (OPN), two noncollagenous bone matrix proteins. The integrin $\alpha_v\beta_3$ was chosen for attachment and migration assays, because it is known to interact with not only these two bone matrix

proteins, but laminin as well. LNCaP and C4-2 cells adhered to all three substrata, but only C4-2 attachment could be inhibited with increasing concentrations of antibodies against $\alpha_v\beta_3$ integrin (Figure 5). At high antibody concentrations of 10 $\mu\text{g/ml}$, attachment of the metastatic C4-2 cells to all three substrata was reduced by approximately 60%, but no attachment effect was seen for the non-metastatic LNCaP cells. The role of $\alpha_v\beta_3$ heterodimer in cell migration was evaluated using modified Boyden chambers, and the haptotactic responses of each cell line quantified in response to laminin, vitronectin, and osteopontin. Figure 6 shows the resulting cell migratory behaviors of LNCaP and C4-2 cell lines on these three bone matrix proteins. In comparison to C4-2 cells, LNCaP cells migrated less on laminin and vitronectin, and failed to migrate on osteopontin (Fig. 6). The migration of C4-2 cells, but not LNCaP cells, was inhibited by an $\alpha_v\beta_3$ isotype-specific integrin antibody (Fig. 6).

Soluble Stromal factors induce C4-2, but not LNCaP, cells to attach to laminin

To begin identifying possible regulators of integrin subunit usage and cell behavior in LNCaP and C4-2 cell lines, we tested the effect of stromal factors on cell line interactions with laminin substrata. Cells were treated with conditioned media from primary cultures of the transitional or peripheral zone stromal cells of the prostate gland, from three different patients with prostatic adenocarcinoma, and were allowed to adhere for 90 minutes. Figure 7A shows the differential effects of this soluble paracrine factor on the spread of LNCaP and C4-2 cells. Although all conditioned media caused C4-2 cells to spread more rapidly on laminin, none had any effect on the spreading of LNCaP cells. No spreading effects were seen for either cell line when treated with conditioned media from mouse fibroblastic cells (Sw3T3 cells) or when left untreated (control).

The effects of conditioned media could be reversed using integrin isotype-specific, function-blocking antibodies. Figure 7B shows that function-blocking antibodies to both α_6 and β_1 inhibit cell spreading in both control and soluble stromal conditioned media-treated cells.

Function-blocking antibodies against the α_3 integrin subunit also were able to block half of the increase in cell spreading induced by conditioned media. The $\alpha_v\beta_3$ specific antibody decreased cell spreading, but only by 30% in control medium-treated cells, and had minimal effect on cells treated with the soluble stromal conditioned media. Quantification of integrin cell surface expression by FACS analyses (Figure 8) did not reveal any change in receptor availability between control and stromal cell conditioned media-treated cells, suggesting that the observed differences in response to external regulation are likely instead to involve regulation of integrin affinity.

Discussion

Cell migration through the basement membrane and surrounding stroma is a fundamental early step in cancerous metastasis. Accordingly, when the normal interactions between prostate epithelium and its microenvironment is altered, genomic instability and metastatic progression of prostatic epithelium can result (41-45). While some changes in matrix composition and metalloproteinases are known to occur late in prostate cancer progression, for example, the loss of collagen VII and the laminin $\gamma 2$ subchain in carcinomas (5, 8), we have focused instead on cell surface changes, and specifically on early shifts in cellular response to environmental cues. We have found shifts in integrin receptor usage among prostate cancer cells to be associated with variations in cell behavior on different substrata and with the presence or absence of soluble stromal factors.

Our findings on integrin usage characteristics in LNCaP cells, and their more invasive, derived cell lines, correlate well with immunohistochemical staining for integrin expression in patient specimens (Table 2; 5-8, 46, 47), and add to a number of past *in vitro* studies showing differences in integrin heterodimer expression among cultured normal, neoplastic, and prostate carcinoma cells (10, 11, 37, 47, 48). Because previous studies of integrin expression in various epithelial carcinomas have yielded conflicting results, attributable to both antibody specificity and the disorganization and/or loss of basement membrane common to poorly differentiated lesions in organs other than prostate, we compare our results to previous results from prostate tissue only. The increase in α_2 expression level between LNCaP and C4-2 cells is similar to one found in prostate lymph node metastases and perineural spaces (6). The shift in α_6 partner choice also agrees with previous studies, in which $\alpha_6\beta_1$ and $\alpha_6\beta_4$ were both found in normal prostate cells, whereas the expression of the β_4 subunit was lost in carcinomas (5, 6, 8). Because the integrin subunit α_6 preferentially associates with β_4 , it is believed that a reduction in β_4 subunit expression results in a relative increase in the formation of the $\alpha_6\beta_1$ heterodimer (49).

We report here that, despite constant integrin β_1 and β_4 subunit *expression* levels, a parallel shift in integrin subunit *use* is in place among parental LNCaP and its derivative cell lines. LNCaP cells attach to laminin primarily with $\alpha_6\beta_4$, while cells in the more invasive C4-2 subline attach with $\alpha_3\beta_1$ and $\alpha_v\beta_3$ (Figs. 2 and 4). In addition to attachment, the $\alpha_6\beta_1$ heterodimer was found to be involved in cell spreading behaviors (Figure 7). Differential expression of the α_6 subunit, rather than β subunits, could account for a varied pattern of integrin heterodimerization. The integrin α_6 subunit exists as two isoforms, α_{6A} and α_{6B} (47, 50-53), both of which are expressed in LNCaP cells (47). While we do not yet know α_6 isoform expression in C4-2 cells, overexpression of the α_{6A} isoform is known to increase $\alpha_6\beta_1$ heterodimerization as well as overall cell motility, tumorigenicity and invasion (13).

Our results show a shift between LNCaP cells and C4-2 cells in the use of integrin heterodimers for laminin attachment, namely $\alpha_6\beta_4$ use declines in C4-2 cells in conjunction with an increased use of $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_3\beta_1$ (Figure 2). Unlike $\alpha_6\beta_4$, which is associated with stable, hemidesmosomal cell attachment sites and appears to restrict cell migration, $\alpha_6\beta_1$ and $\alpha_3\beta_1$ both are involved in the formation of dynamic focal contacts important for cell locomotion (13). Prostatic cell lines able to form invasive tumors in immunocompromized mice have previously been shown to have increased expression of the $\alpha_6\beta_1$ heterodimer (10, 13, 37, 54), and antibodies against $\alpha_6\beta_1$ are able to inhibit invasion. In agreement with Vafa et al. (1998), we also found the $\alpha_6\beta_1$ heterodimer to be more involved in cell spreading than static cell attachment (Figs. 2 and 7). The intrinsic involvement of this heterodimer in the interpretation of environmental cues, such as stromal factors by C4-2 cells, remains to be studied further. The $\alpha_3\beta_1$ integrin is likely to have both direct and indirect effects on cell motility, because of its bi-directional interactions with the matrix. The known ability of $\alpha_3\beta_1$ to affect laminin chains and overall basement membrane architecture (55, 56) is particularly suggestive, given that proteolytic cleavage of laminin can drive cells from static adhesion to active migration (57, 58). It is interesting to note in this context that

past studies of oncogene-transformed rat prostate cells (37, 54) have found overexpression of both laminin type I and $\alpha_6\beta_1$.

An additional integrin heterodimer implicated in increased metastatic potential and tumorigenicity is $\alpha_v\beta_3$ (59-61). Although not frequently found in epithelial cells, $\alpha_v\beta_3$ is common to a number of bone-receding metastases, including prostate and breast carcinomas (10, 12, 62). In the LNCaP model system, $\alpha_v\beta_3$ was similar to $\alpha_3\beta_1$, in that its individual subunits were expressed at all stages of cancerous progression (that is, in all cell lines), but the assembled, functional heterodimer was only detectable in the more metastatic cell lines C4, C4-2 and C4-2B (Table 1; Fig. 4). Although such differences in integrin usage have been noted before between very different cell lines with different metastatic potentials, this is the first study we know of that reveals shifts in integrin *usage* between cells with common genetic backgrounds but different metastatic potentials.

Two potential consequences of $\alpha_v\beta_3$ heterodimer usage in the metastatic LNCaP sublines are 1) preferential relocation to the bone and 2) increased cell survival/suppressed cell death. Integrins are likely to be involved in both the establishment of prostate cell anchorage to the bone endothelium and its surrounding matrix, as well as the transmission of multiple cues from the cells' microenvironments, supporting cell survival and proliferation. Not only do C4-2 cells, cells known to preferentially relocate to bone integrin (27), increase their use of the $\alpha_v\beta_3$, but we show here that they use this integrin to migrate on osteopontin, a key component of bone matrix. $\alpha_v\beta_3$ has also previously been shown to support migration on vitronectin, another dominant component of bone matrix (63, 64). Regardless of the role of $\alpha_v\beta_3$ in binding metastatic cells to the bone matrix, this integrin heterodimer is a good candidate for helping cells escape adhesion-dependant limits on cell survival. Although loss of appropriate adhesion is normally a cue for apoptosis, human breast cancer cells are able to use $\alpha_v\beta_3$ to inhibit p53 activity and suppress the

cell death pathway (65). Likewise, $\alpha_v\beta_3$ has been shown to regulate cell proliferation in prostate epithelia (66).

Integrin involvement in prostate epithelial proliferation is likely to be based, at least in part, on interactions between integrins and growth factor receptors, used by the cells to interpret positive and negative growth factor and cytokine signals coming from surrounding stromal cells (1, 67 and references within). Certainly, growth factors and integrins share common signaling cascade components (for example the small GTPases), but there is also evidence that the two types of surface proteins may associate directly and preferentially with one another (67). In the context of changing integrin usage (such as we see between LNCaP and C4-2 cells or has previously been reported *in vivo*), preferential associations between the growth factor receptors and the changing integrin heterodimers could have serious consequences for the cells' responses to environmental cues. Indeed, Figure 7 shows that C4-2 and LNCaP cells do respond differently to soluble factors in media from prostate stromal cells, with only C4-2 cells showing increased spreading on laminin substrates after stromal media treatment.

We investigated the roles of $\alpha_6\beta_1$ and $\alpha_v\beta_3$ integrins in stromal response, by adding function-blocking antibodies against these integrins to C4-2 cell cultures before and after treatment with stromal media. The dramatic increase in C4-2-laminin spreading following stromal media treatment was relatively unaffected by $\alpha_v\beta_3$ function-blocking antibodies, whereas antibodies against either α_6 or β_1 completely obliterated spreading on laminin both before and after stromal treatment (Figure 7) -- a result in agreement with Vafa et al.'s 1998 work on c-erb B2/neu transformed rat prostate epithelial cells. The identities of the responsible soluble factors (cytokines, growth factors, or other) remain to be determined. Our results raise the possibility that the $\alpha_6\beta_1$ -specific response to stromal factors is due to direct interactions between growth factor receptors and integrins, and/or indirect involvement of such cascade components as the small GTPases.

In summary, use of a lineage-derived LNCaP cancer cell progression model has allowed us to compare the integrin expression levels, heterodimer usage, and cell behaviors of cells sharing a common genetic background, but differing in metastatic potentials, on different matrices in the presence or absence of stromal factors. We have found that, although integrin expression levels do not change markedly among the cell lines (with the exception of an increase in collagen-binding α_2 expression), integrin heterodimer usage does change, especially usage of $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_v\beta_3$. While all cells attached to laminin, vitronectin and osteopontin matrices, only the more metastatic cell lines (C4-2 in particular) were able to migrate on osteopontin. C4-2 cell behaviors were also unique because of their response to prostate stromal cell-derived factors. The striking increase in the spreading of C4-2 cells on laminin following treatment with stromal factors could be completely obliterated by addition of function-blocking antibodies against α_6 or β_1 , but not α_2 , α_3 , β_4 or $\alpha_v\beta_3$. Because C4-2 cells were found to increase usage of $\alpha_6\beta_1$, while decreasing usage of the $\alpha_6\beta_4$ heterodimer, further studies are called for to characterize this shift in heterodimer usage, and its direct and/or indirect effects on cell behavioral and survival responses to matrix and stromal environmental cues.

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Figure Legends

Table 1. Integrin expression in LNCaP and its more metastatic, derived C4-2 cell line. Values for integrin expression are presented as the mean of two individual experiments with the range given in parentheses. An isotype non-specific antibody was used for control. All experimental fluorescence values are reported as the ratio of the control and specific fluorescence values.

Table 2. Comparison of previously reported integrin usage during prostate cancer progression and the usage found in the LNCaP progression model.

Figure 1. Vinculin and actin staining in LNCaP and C4-2 cell lines. Both LNCaP and C4-2 cells show diffuse actin staining and small actin fibers, when labeled with Phalloidin. Numerous focal adhesions (white arrows) are also visible in both cell lines, when cells are stained for vinculin.

Figure 2. Inhibition of cell attachment to a laminin substrate, using integrin subunit-specific anti-bodies. LNCaP and the derived sublines C4, C4-2 and C4-2B were pre-incubated with function blocking antibodies as marked. Experimental attachment is shown as a percentage of control, untreated cell attachment, and results are presented as the mean of triplicate experiments, with standard deviations shown as error bars.

Figure 3. Fluorescence Activated Cell Sorting (FACS) analyses of surface expression of the individual, laminin-binding integrin isotypes β_1 , β_3 and β_4 . Exemplar curves are shown for each fluorescently-labeled (FITC) beta subunit antibody, and its appropriate, non-specific IgG control antibody. For a complete listing of all isotypes tested see Table 1.

Figure 4. Immunoprecipitation of biotinylated cell surface integrin isotypes using α_3 and β_1 (A), α_6 (B) and $\alpha_v\beta_3$ (C). Retrieved complexes from each LNCaP, C4, C4-2 or C4-2B cell line were separated by polyacrylimide gel electrophoresis, under reducing conditions, blotted, and visualized with peroxidase-conjugated streptavidin.

Figure 5. Antibody-mediated attachment inhibition of LNCaP and C4-2 cells on vitronectin or osteopontin substrata. Inhibition of attachment is shown with increasing $\alpha_v\beta_3$ antibody concentration expressed as a percentage of control, untreated cell attachment. Values are the mean of triplicate experiments and error bars represent standard deviations.

Figure 6. Migration of LNCaP and C4-2 cells on laminin, vitronectin or osteopontin substrata, in the presence and absence of 10 $\mu\text{g/ml}$ control or $\alpha_v\beta_3$ function-blocking, integrin antibody. Boyden chambers were used for haptotactic assays, and values shown are the average of triplicate experiments. Error bars represent standard deviations.

Figure 7. The effects of stromal-cell conditioned media on LNCaP and C4-2 cell attachment to laminin substrata. (A) LNCaP and C4-2 cell attachment responses to conditioned media from four primary prostate stromal cell cultures (3 different patients). Cells with pronounced lamellipodia were scored as "attached." All % attachment values are normalized to the behavior

of Control LNCaP, and C4-2 cells, that were not treated with conditioned media (after 90 minutes, 25% of C4-2 untreated, control cells had attached, compared to only 6% of LNCaP untreated, control cells). (B) Comparison of C4-2 cell attachment %'s following treatment with conditioned stromal media, in the presence or absence of function-blocking integrin isotype-specific antibodies. All experiments were repeated six times.

Figure 8. FACS analysis of cells treated with stromal conditioned media. Surface expression of integrin subtypes after 90 minutes treatment with stromal conditioned media. No change in the integrin surface expression is evident.

Expression of Integrins by FACS Analysis

	<u>LNCaP</u>	<u>C4-2</u>
α_2	7.7 (2.6)	13.7 (0.4)
α_3	4.4 (0.6)	3.4 (1.2)
α_6	9.8 (0.4)	10.8 (0.8)
α_v	15.8 (0.8)	14.4 (1.4)
β_1	17.2 (3.2)	15.5 (2.8)
β_3	2.4 (0.8)	2.6 (0.4)
β_4	1.7 (0.6)	1.3 (0.2)

Table 1. Edlund et al.

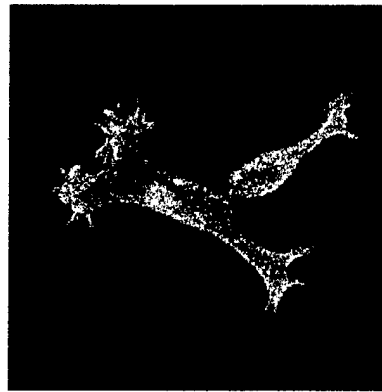
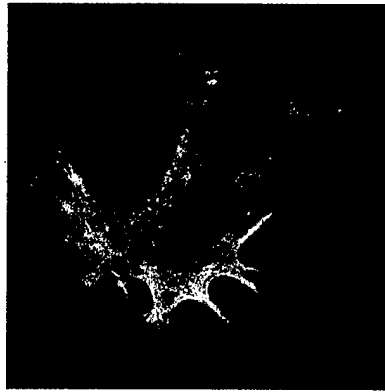
Integrin heterodimer	LNCaP	C4-2	Normal to Carcinoma
$\alpha_3\beta_1$	+	++	No change ^{3,6}
$\alpha_6\beta_1$	+	++	No change ^{2,3,4,6}
$\alpha_6\beta_4$	++	-	Decreases ^{1,4,5}
$\alpha_v\beta_3$	-	+	Increases ⁷

¹Allen et al., 1998, ²Bonkhoff et al., 1993, ³Cress et al., 1995, ⁴Knox et al., 1994, ⁵Nagle et al., 1994, ⁶Nagle et al., 1995, ⁷Zheng et al., 1999

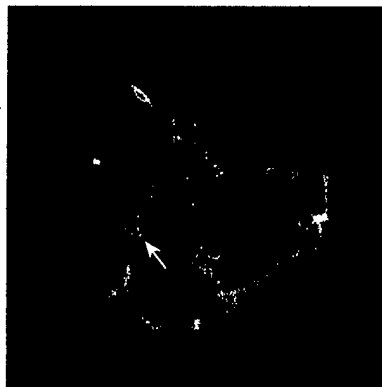
Table 2. Edlund et al.

LNCaP

C4-2



Actin



Vinculin

Figure 1. Edlund et al.

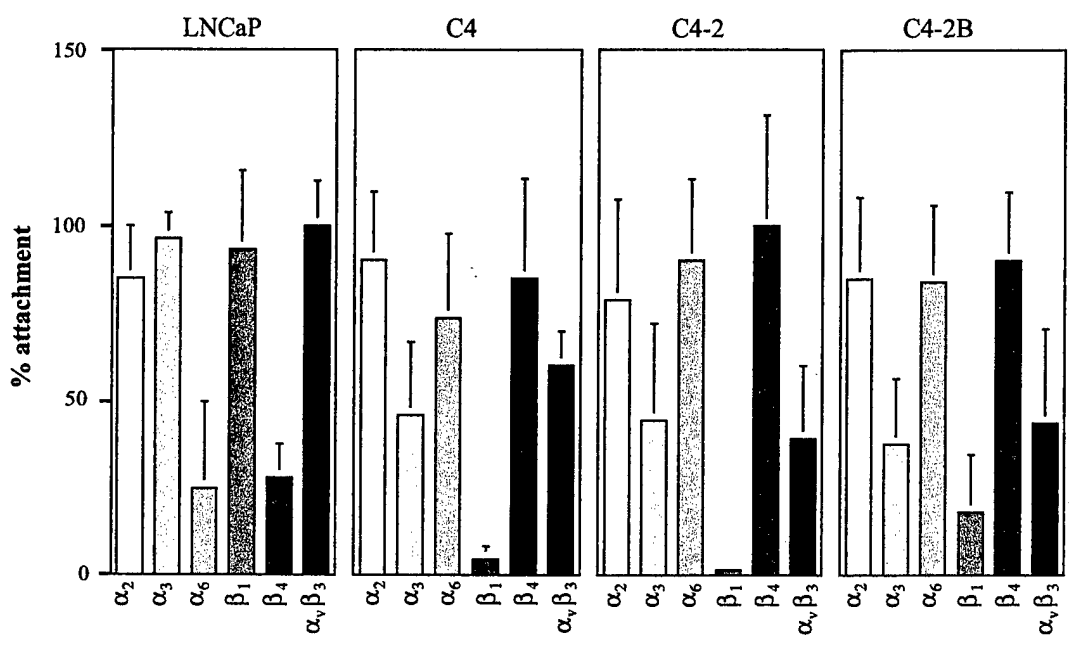


Figure 2. Edlund et al.

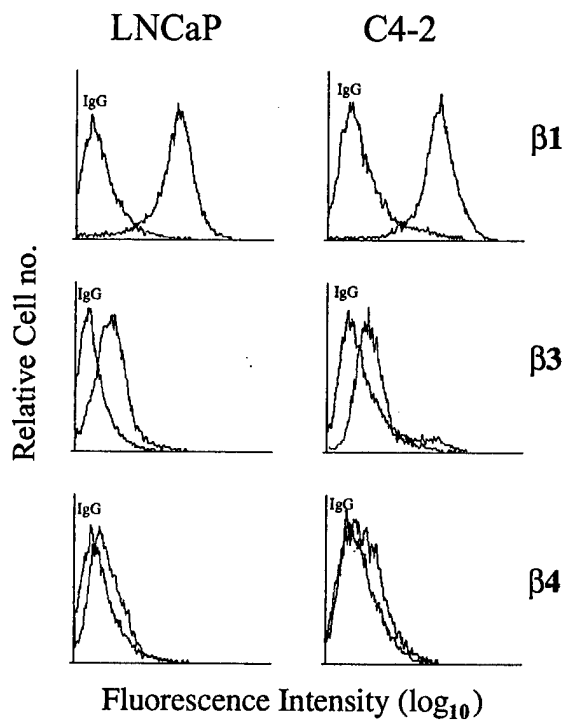


Figure 3. Edlund et al.

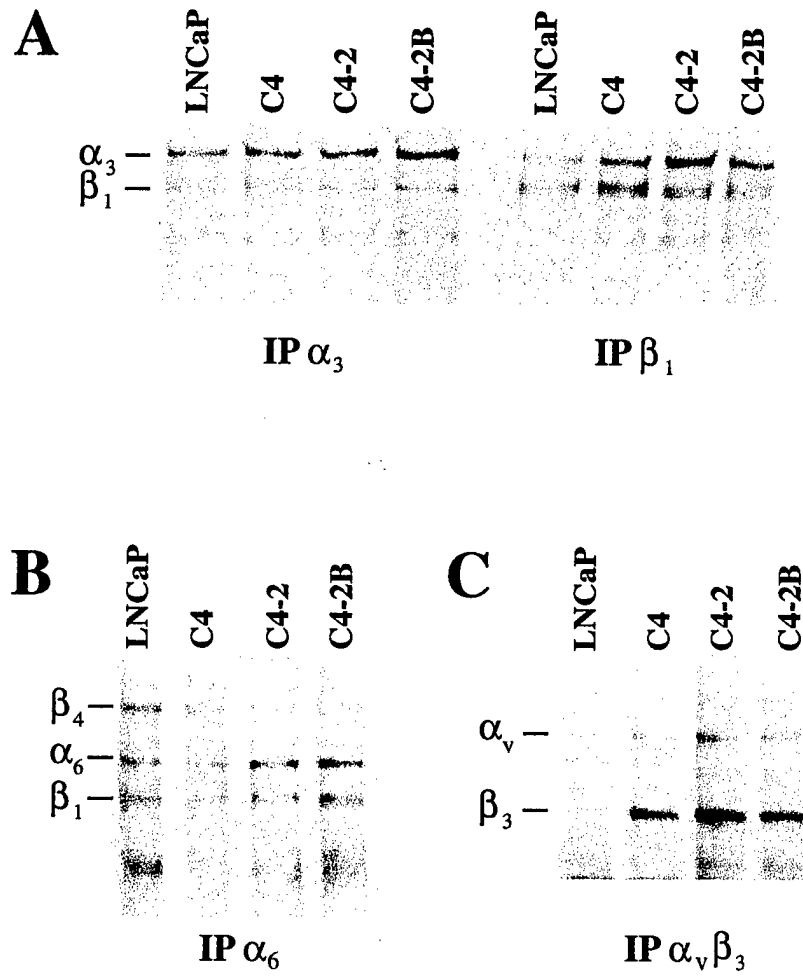


Figure 4. Edlund et al.

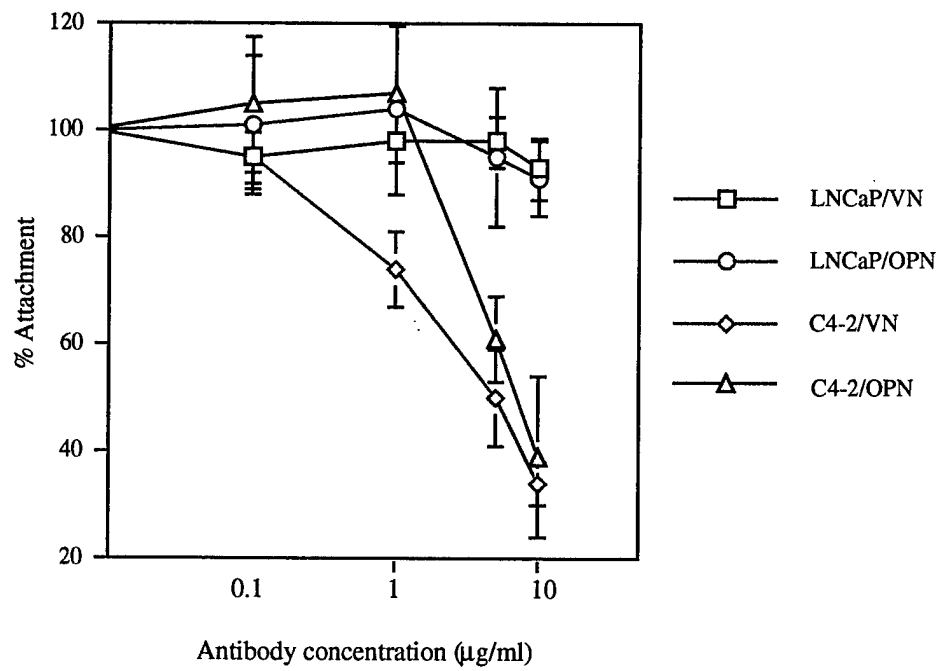


Figure 5. Edlund et al.

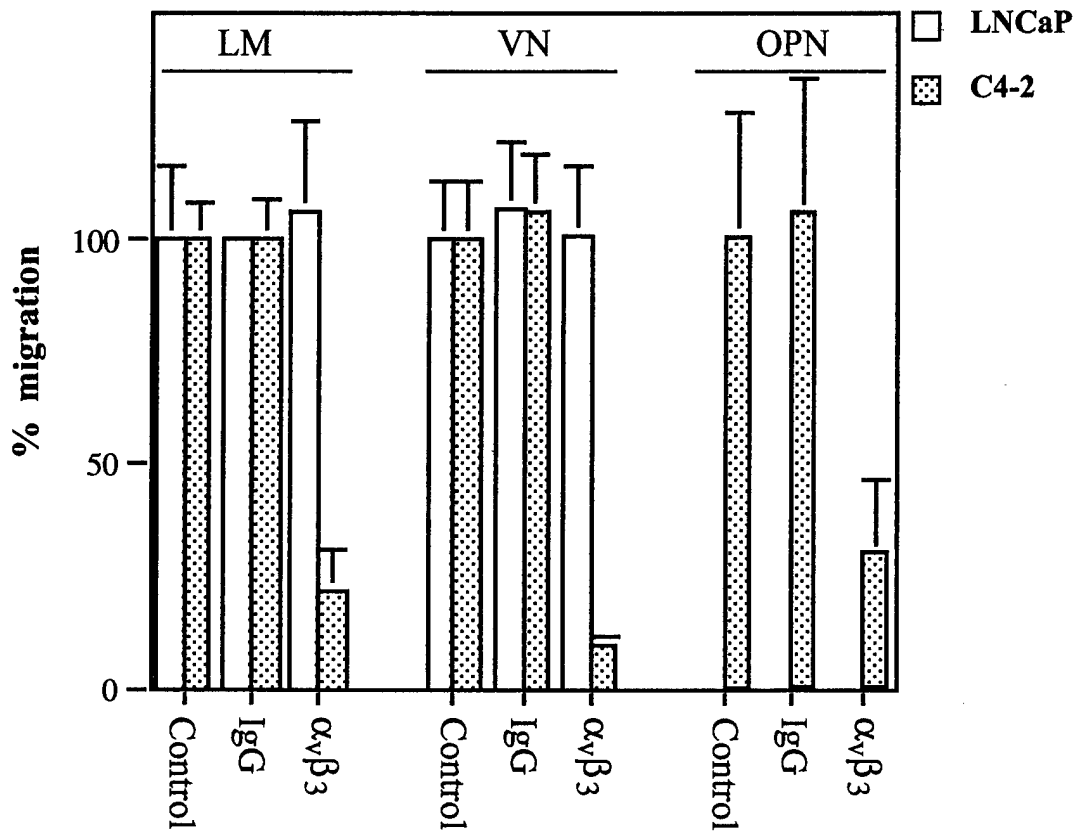


Figure 6. Edlund et al.

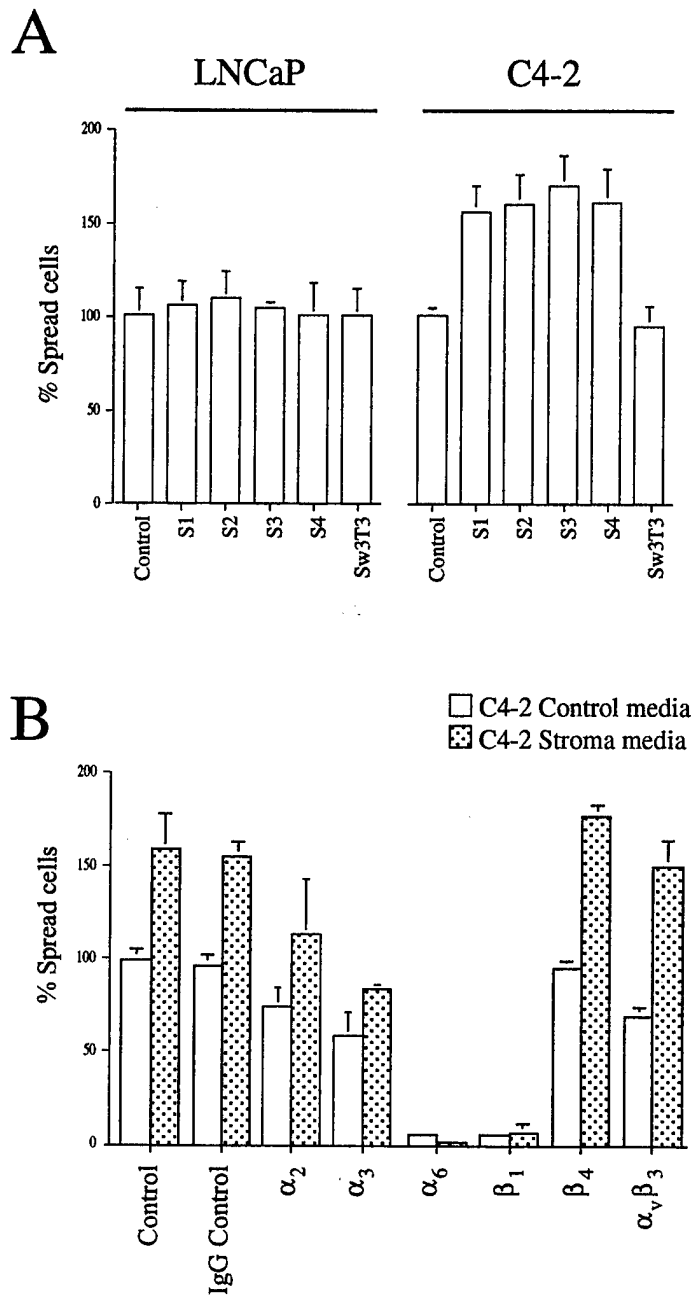


Figure 7. Edlund et al.

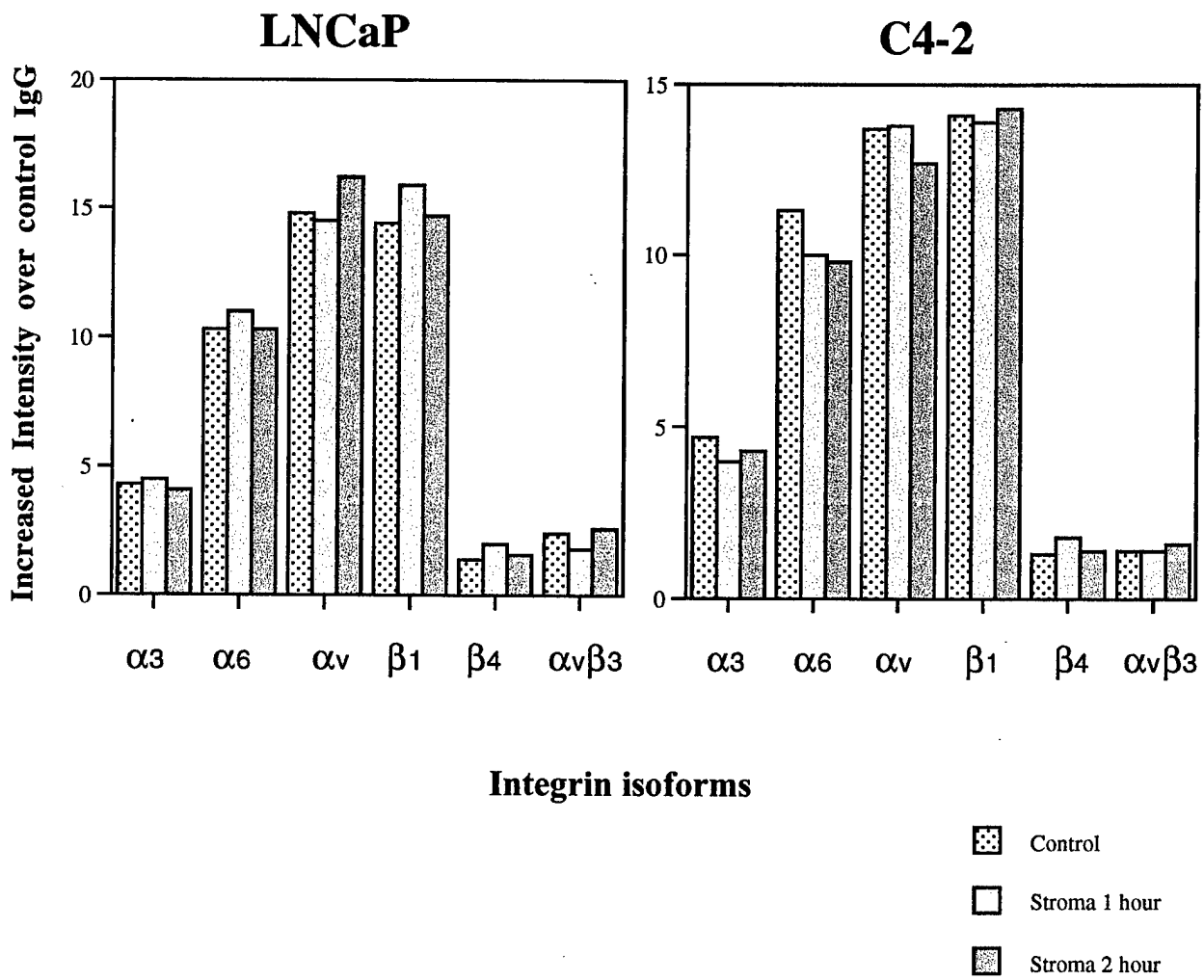


Figure 8. Edlund et al.