Prostate Cancer Cell Adhesion to Bone Marrow Endothelium: The Role of Prostate-Specific Antigen

Victor I. Romanov, Terry Whyard, Howard L. Adler, Wayne C. Waltzer, and Stanley Zucker

Departments of Medicine and Urology, State University of New York at Stony Brook, Stony Brook, New York, and the Veterans Affairs Medical Center, Northport, New York.

ABSTRACT

Bone metastasis is the most frequent complication of prostate cancer (PC). Elucidation of the biological basis of this specificity is required for the development of approaches for metastatic inhibition. We investigated the possibility that the preferential attachment of PC cells to bone marrow endothelium (as opposed to endothelium from other organs) affects this specificity. We selected, from peptide phage-displayed libraries, peptide ligands to surfaces of PC cells (C4-2B) attenuated (30–40%) binding of C4-2B cells to bone marrow endothelial cells (BMECs). We then determined the molecules on the surface of C4-2B cells interacted with the selected peptides using column affinity chromatography and a cDNA expression phage-displayed library generated from C4-2B cells in T7 phage. We identified a phage from the cDNA library that specifically bound to one of the selected peptides-L11. This phage displayed the amino acid sequence homologous for the COOH-terminal portion of prostate-specific antigen (PSA). To examine the possible direct involvement of PSA in the interactions between PC and BMECs, we performed a cell–cell adhesion assay. Antibodies to PSA attenuated PC cells adhesion to BMECs. In addition, exogenous proteolytically active PSA modulated this adhesion. Finally, inactivation of mRNA coding PSA by a small interfering RNA (siRNA) diminished C4-2B cell adhesion to BMECs. These results indicate that PSA expressed as secreted and surface-associated molecules in C4-2B cells is involved in cell–cell interactions and/or digest components of bone marrow endothelium for preferential adhesion and penetration of PC cells. The suggested experimental approach is a promising strategy for identification of cell surface molecules involved in intercellular interactions.

INTRODUCTION

The “seed and soil” theory of Paget, postulates that the microenvironmental advantages in the target tissue favors the residency of cancer cells in this tissue (1).

During the metastatic process, cancer cells must cross the endothelial barrier. On the basis of the demonstration of preferential adhesion of prostate and breast cancer cells to bone marrow endothelial cells (BMECs) as compared with human umbilical endothelial cells, Lehr and Pienta proposed that selective metastasis to bone might be the result of enhanced attachment to the bone marrow endothelium (as opposed to endothelium from other organs; Ref. 2). The same group subsequently elucidated some features of this process. Because bone marrow endothelium is fenestrated, it is possible that cells might directly pass through it and interact with the underlying matrix, but not with the surfaces, of endothelial cells. However, it has been demonstrated by scanning electron microscopy that prostate cancer (PC) cells (PC-3 cell line) interact directly with endothelial cell surfaces but not with underlying extracellular matrix (3). These intercellular interactions are mediated in part by tumor cell surface proteins and in part by the endothelial cell receptors (3).

Many proteins responsible for the interactions of cancer cells with endothelium have already been identified. Selectins, integrins, cadherins, and immunoglobulins, as well as some unclassified molecules, have been shown to determine interactions between cancer cells and endothelium. Selectins contribute to the initial contact of circulating cells with endothelium, whereas integrins play a role in the development of more stable attachment (4).

Several adhesion molecules, specific for particular cancer cell types, have been recently identified. For instance, adhesion of PC cells to BMECs was inhibited by a galactose-containing carbohydrate (2). The tumor-associated carbohydrate-Thomsen-Friedenreich antigen (T antigen) expressed on breast and prostate carcinoma cells and its counter partner a β-galactoside-binding protein, galectin-3, expressed on the endothelium cells have been implicated in tumor cell adhesion to endothelium (5). Furthermore, not only heterotypic adhesion of metastatic cells to endothelium, but also homotypic aggregation at the sites of their primary attachment to endothelium mediated largely by interaction of T glycoconjugant with galectin-3 (6, 7). In addition, endothelium became activated by cancer-associated carbohydrate structures such as T antigen expressed on circulating glycoproteins and neoplastic cells (8). It was also showed that antibodies to CD11a, CD18, LFA-1, and CD31 inhibited the binding of PC cells to bone marrow endothelium, showing that cell–cell adhesion is a complicated cooperative process (2).

Our goal has been the identification of additional specific cell–cell adhesion molecules and mediators of bone endothelium–PC cell interactions. To achieve this aim, we systematically selected PC cell-specific peptide ligands that attenuated the binding of PC cells to BMECs. Using a cDNA expression phage display library, we identified T7 phage that bound to one of the biologically active peptides selected from random peptide library. We identified the amino acid sequence of the selected phage as prostate-specific antigen (PSA). Blocking of the PSA resulted in diminished adhesion of PC cells to BMECs.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Peptides. Human PC cell lines LNCaP, PC-3, and DU145 were obtained from American Type Culture Collection (Manassas, VA). BPH-1, prostate epithelial cells (derived from a prostate with benign hyperplasia and immortalized with SV40) were a kind gift of Dr. Simon Hayward, University of California at San Francisco, San Francisco, CA. These cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics (Life Technologies, Inc., Gaithersburg, MD). C4-2 and C4-2B cell lines, derivatives of LNCaP cells, were from UroCor (Oklahoma City, OK) and were maintained in T-medium according to the recommendations of Dr. L. W. K. Chung (line contributor), Emory University, Atlanta, GA. Normal prostate epithelial cells were obtained from Cambrex (East Rutherford, NJ) and were maintained in the prostate epithelial cell medium provided by Cambrex. As an in vitro model of bone marrow endothelium, we used immortalized human BMECs. These cells obtained as a kind gift from Dr. Graca Almeida-Porada (University of Reno, Reno, NV) have characteristics that are indistinguishable from primary cultures of BMECs (9). BMECs were maintained in M199 medium with supplements as described previously (9). All of the cells were grown in a CO2 incubator at 95% air/5% CO2 and 37°C.
Polyclonal anti-PSA antibodies were obtained from DAKO (Carpinteria, CA). Anti-M13 polyclonal biotinylated antibodies were a gift from Dr. V. Petrenko (University of Auburn, Auburn, AL). Anti–T7 Taq-horseradish peroxidase (HRP)-conjugated monoclonal antibodies were purchased from Novagen (Madison, WI). Peptides were synthesized by BioPeptide (San Diego, CA) or by Multiple Peptide Systems (San Diego, CA) and were purified by high-performance liquid chromatography to >95%; the molecular weights were confirmed by mass spectroscopy. Enzymatically active PSA was purchased from Cortez Biochem (San Leonardo, CA).

Random Peptide Phage Display Libraries. In this study, we used the landscape library R8–R11mer, which was designed by the insertion of random 8 amino acids in NH2-terminal of pVIII coat protein as described previously (10) and the R8–15mer library that was a gift from Dr. George Smith (University of Missouri, Columbia, MO). This library was generated by the insertion of random 15 amino acids in ~300 copies of pVIII. The diversities of both libraries were in the range of 109–1010 independent clones, and ~100 copies of each clone were applied in the first round of biopanning.

Biopanning with Random Peptide Phage Libraries. Biopanning was performed as described previously (11). The ratio of output/input (number of cell-associated phage divided by the numbers of total phage applied to the cells) was calculated for each round. The final round of biopanning was defined after this parameter reached the value of 0.5–1.0. The sequences of DNA coding inserts were determined for 48 of the selected phage from the output of the last round of biopanning as described previously (11). Translated peptide sequences were aligned for further analysis.

Confirmation Phage-Binding Assays. Immunofluorescence labeling of bound phage was performed according to the procedure described by Barry et al. (12) with some modifications. We used anti-M13-biotin conjugated polyclonal antibodies and streptavidin–FITC as secondary antibodies. For binding experiments, cells were incubated with phage for 2 h at 4°C and were fixed immediately by 4% buffered paraformaldehyde for 15 min at 4°C.

Database Search. Matches of peptide consensus sequences with molecules in protein databases were determined using the BLAST program. Protein query versus protein and translated databases as well as translated query versus protein databases were performed. Matched proteins were considered for further investigation depending on the level of their homology with tested sequences and the biological relevancy of matched proteins. Eukaryotic proteins with a homology of >60% that might theoretically serve as soluble ligands for receptors on the surface of target cells or might be expressed on the surface of potential cellular partners for cell–cell interactions with target cells were considered as candidates for further investigation.

Cell–Cell Adhesion. BMECs or human umbilical vein endothelial cells were grown as monolayers in a 96-well plate. Cells assayed for the attachment were considered as candidates for further investigation.

Cell Survival and Apoptosis Assays. C4-2B cells plated on tissue culture plastic with selected peptides were assayed for survival by the trypan blue exclusion assay and by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining for the identification of necrotic and/or apoptotic cells. Briefly: 5000 cells/well were plated in 24-well clusters with 100 nm-10 μM of tested peptide in 1 ml of growth medium and were incubated at 37°C in CO2 incubator. Twenty-four h later, cells were assayed by the trypan blue exclusion test. To identify apoptotic cells, DAPI staining (Aldrich Chemical Co., Milwaukee, WI) was used. Cells were prefixed with ice-cold methanol-acetone 1:1 for 10 min, stained with 8 μg/ml of DAPI in PBS for 20 min at room temperature, washed with PBS, air-dried, and analyzed by fluorescence microscopy. Condensed and fragmented nuclei, typical for apoptosis, were easily distinguishable from intact nuclei. Motility (Chemotaxis) and Invasion Assays. Motility and invasion assays have been performed as described previously (11). Three Transwell inserts were used for each experimental condition.

RNA Interference (RNAi) Experiments. The siRNA sequence targeting human PSA chosen in this study (from mRNA sequence; GenBank accession no. BC-056665) corresponds to the coding region 787–1230. Computer analysis using the software developed by Ambion Inc. confirmed this sequence to be a good target. The double strand RNA was prepared using in vitro transcription and was digested by RNaseIII (Cocktail kit; Ambion Inc., Madison, WI). Short RNAs were transfected using DOTAP reagent according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). Unless otherwise described, transfection used 20 nm RNA duplex in 0.5 ml of RPMI 1640 containing 5% FCS. Fragment of RNA coding GAPDH has been used as a negative control under similar conditions (Ambion Inc.). Conditioning media from PSA siRNA, GAPDH siRNA, and mock-transfected C4-2B cells were concentrated by Centricon units (cutoff, 10K) or were precipitated with tri-chloroacetic acid (TCA) and prepared for electrophoresis. Western blotting was performed as described previously (13). For immunofluorescence experiments, siRNA-transfected cells were cotransfected with EGF-containing plasmid (Clontech, Carlsbad, CA) to determine transfection efficiency. PSA or GAPDH were observed in transfected cells by fluorescence microscopy using specific antibodies and TexasRed-conjugated secondary antibodies as described previously (11).

Three-Dimensional Cultures. Fifty μl of Matrigel (0.3 mg/50 μl) per well were added into 96-well plates, and plates were placed in a 37°C CO2 incubator for 1 h to allow matrix to form gel. C4-2B cells were plated at 1000 cells/well in 200 μl of complete or serum-free RPMI 1640. Alternatively, cells (PC-3 in all of the experiments and C4-2B cells in some experiments) were directly embedded in the Matrigel. To test the ability of the selected peptides to modify three-dimensional cluster morphology, peptides were added three times during the growth to the incubation medium at concentrations of 100 nm, 5 μM, and 15 μM in 50 μl of RPMI 1640. Three wells were used for each individual peptide titer. Nonrelevant peptide was used as a control. The medium with or without peptides was replaced every 48 h. Cultures were routinely grown for 12–14 days. After 5, 7, and 12 days, cultures from some wells were washed gently with PBS and fixed in 2.5% buffered paraformaldehyde for 15 min. Samples were investigated for general morphology by phase contrast microscopy first and by confocal microscopy for lumenal structures as described previously (14).

T7 cDNA Library. The cDNA expression library in T7Select10–3b vector was constructed based on the manufacturer’s recommendations using the T7Select phage display system (Novagen, Madison, WI). The source of RNA for cDNA synthesis was C4-2B cells. Fifteen 60-mm diameter culture dishes with 70–80% confluent C4-2B cells were used for the total RNA purification with RNAeasy mini kit (Ambion, Austin, TX). Eight μg of mRNA were purified from total RNA using the Poly(A) purist kit (Ambion). The cDNA was synthesized using random primers and the Orient Express kit (Novagen). The library was amplified by infecting 50 ml of log phase BLT5615 with the library portion. Insert sizes were analyzed by PCR using phage plaques as templates and T7UP, T7DOWN primers as described in the T7Select system manual. The PCR products were run on a 2% agarose analytical gel; 5 μl of reaction volume were treated with enzymes from ExoSap kit (Amersham Biosciences, Piscataway, NJ) and were sequenced using T7UP primer. The library was stored as aliquots at −78°C.

Column Affinity Chromatography with Peptide/Phage and cDNA Library. Two peptides with profound inhibition activity (L11, F28) were chosen as primary targets. Peptides were immobilized on HiTrap NHS-activated columns according to the manufacturer’s protocol (Amersham, Biosciences). Twenty-five-milliliter aliquots of T7 cDNA library were poured over each column, followed by washing with PBS containing 0.1% Tween 20 (PBS-T). The elution step was performed with PBS containing 1% SDS. Eluates were amplified as described by the T7Select system manual (Novagen) and were applied for the final round of biopanning. The selection process was monitored by random sequencing of several clones in intermediate steps and by calculating the out:in ratio as described for the peptide phage libraries. After the final fifth round of biopanning, DNA from 20 clones for each target was PCR amplified and sequenced as described above.

Analysis of Matches. Sequences of the selected phage were translated into amino acid code and analyzed by searching GenBank protein databases.
Identified matches were divided into three categories. The first group included matches with known proteins that may be expressed on or may be associated with the cell surface of prostate epithelial cells. The second group included identified human proteins with yet unknown functions. The third group included expressed sequence tags (ESTs) that were derived from libraries prepared from human epithelial tumors. We considered for the immediate investigation only phage-displaying amino acid sequences matching previously characterized proteins in frame with 10b phage protein.

**T7 Phage-Binding Confirmational Assays.** To confirm the specificity of the binding of the selected T7 phage to target peptide, two types of assays were performed. For the first assay, we used monoclonal antibodies generated against part of the major capsid protein of T7 to localized phage bound to the L11 peptide. L11 peptide was adsorbed to the plastic by overnight incubation of 100 μl of L11 peptide per well at a concentration of 20 μM Maxi-Sorb in 96-well plates. After three washes with PBS-T and blocking with PBS containing 0.1% BSA (PBS/BSA) selected phage (10⁸ colony-forming units in 96-well plates) was applied. Detection was performed by incubation with anti-T7 Tag-HRP conjugate (Novagen), followed by the addition of HRP substrate. In the inhibition experiments, phage, before addition to immobilized L11, were preincubated with 50 μg/ml of peptide L11 or with anti-PSA polyclonal antibodies. A second assay was performed similarly, but, instead of the detection of the bound phage with antibodies, competent bacteria were directly added to the phage immobilized by peptide for infection. Infected bacteria were diluted and plated, and plaques were counted to calculate phage titer.

**Selected Peptides-PSA Adhesion Assay.** To confirm the specificity of the interaction of PSA with peptide used as a target for the selection of T7 clones we performed ELISA type assay. Peptide L11 (used for PSA-coding T7 phage isolation), L19, F23, and F28 at concentration 20 μM were immobilized on Maxi-Sorb 96-well plate as described for T7 binding assay. After two washes with PBS-T, wells were blocked with PBS/BSA. PSA was added at concentration of 5 ng/ml and was incubated for 2 h at room temperature. After three washes with PBS-T, immobilized PSA was detected by sequential incubation with polyclonal anti-PSA antibodies, washes with PBS-T, and incubating with secondary HRP-conjugated antibodies. After incubation with HRP substrate, absorbance at 450 nm was measured in a Bio-Tek (Winooski, VT) microtiter plate reader.

**Statistical Analysis.** Results are expressed as mean ± SE of at least three observations. The data shown are representative results observed in triplicate or more experiments. Statistical significance was determined by use of Student’s t test and the SigmaStat program (Jandel Scientific, San Rafael, CA).

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**RESULTS**

**Peptide Selection.** The biopanning procedure was performed as described previously (11) except for omission of the subtractive step, because nonmalignant prostate epithelial cells attach to the BMEC monolayer with an affinity similar to that of C4-2b cells (Fig. 1). Enrichment of the output phage pool with the specific binders from round to round was observed for biopanpings with both libraries and reached at the last round (fourth) 0.5% for biopanning with landscape and 0.1% for f8–15 library.

Forty-eight phage from the final fourth round of the selection were randomly chosen for the sequencing and analysis from both peptide phage libraries. Inserts for the phage selected from the landscape library revealed conserved consensus sequence (DPR) on their NH₂-terminal end (Table 1, underlined). Sequences of the phage selected from f88–15mer library did not show such an evident consensus, but many matches between individual phage including the presence of Pro-Arg (PR) sequence of NH₂-terminal end of inserts for some phage have been observed (Table 1). After exclusion of phage with identical sequences, wild-type phage and phage with compromised sequences, 30 phage with unique sequences of the insert from a landscape library, and 32 from the f88–4 library were chosen for further investigation.

**PC Cell Adhesion to BMECs.** Several cell types were assayed for their ability to attach to BMEC (Fig. 1). This included human PC cells: PC3 derived from bone metastasis; DU145 originated from brain metastasis; LNCaP derived from lymph node metastasis; their derivatives C4-2 and C4-2b; primary culture of normal prostate epithelial prostate epithelial cells; and nontumor BPH cells. We assayed cell adhesion to BMECs in comparison with the attachment to human umbilical vein endothelial cells. All of the assayed cells attached with higher binding affinity to BMECs than to human umbilical vein endothelial cells (Fig. 1).

We next examined the ability of the selected phage to block the attachment of PC cells to BMECs because the determination of peptides’ inhibiting ability of the prostate metastatic cells to interact with BMEC was a major goal of the current study. Each selected phage from both pools (30 + 32) was examined at three concentrations (100, 1,000, and 10,000 colony-forming units/target cell). On the basis of the results of these experiments, we selected for further investigation nine phage that revealed the highest inhibitory activity (Fig. 2). Selected phage inhibited C4-2b attachment to BMECs by 50–60% at a concentration of 10⁸ phage per PC cell (Fig. 2). Inhibitory activity of phage against DU-145 and BPH cells was also observed (data not shown). PC-three-cell adhesion to BMECs was not affected by the selected phage.

Cognate peptides were synthesized based on the sequences of the selected phage inserts. The sequences of synthetic peptides include adjacent amino acids from pVIII protein (Table 1, italic letters). We assayed synthesized peptides for the ability to inhibit attachment of PC cells to BMECs. Peptides were examined in the concentration

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**Table 1 Sequences of inserts for nine selected phage with the highest inhibitory activity chosen for the synthesis of cognate peptides**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L11</td>
<td>AVDPRVAQESPDPAKA</td>
</tr>
<tr>
<td>L19</td>
<td>ADPRAGLQDPAPAKA</td>
</tr>
<tr>
<td>F01</td>
<td>VRDRMGTTPGSMPAE</td>
</tr>
<tr>
<td>F06</td>
<td>FMVLSLQHPAFSNPRAE</td>
</tr>
<tr>
<td>F12</td>
<td>PKTAKGAHHYGPIECPAE</td>
</tr>
<tr>
<td>F14</td>
<td>PRWLPITPSLSILMGMPAE</td>
</tr>
<tr>
<td>F23</td>
<td>LRMFRTRSTSKVLPYPAE</td>
</tr>
<tr>
<td>F28</td>
<td>KDSTTLTNYLELTFYRPDAE</td>
</tr>
<tr>
<td>F38</td>
<td>GPDEVFLFYFVFPYPAE</td>
</tr>
</tbody>
</table>

* Underlined letters conserved consensus sequence (D-P-R); italic letters, adjacent amino acids from p VIII page protein.

1. L, f8-8mer landscape library; F, library f88-15mer.
other potential activities of the selected peptides. This included evaluation of the ability to alter cell proliferation and/or to induce apoptosis, and to modulate the motility and invasion of C4-2b cells. None of these cellular functions was affected by incubation with selected peptides.

Identification of Peptides That Revert Morphology of PC Cellular Clusters in Three-Dimensional Cultures to Acinar Phenotype. We assayed selected peptides by their ability to revert the morphology of disorganized clusters formed by PC cells to an acinar-like phenotype. Because these peptides were selected as cell surface-binding functional ligands, they might be valuable candidates as reagents for modulation of tissue architecture. We first examined the growth pattern of several PC cells as three-dimensional cultures in Matrigel. C4-2b cells were chosen because the selected peptides bound these cells with the highest specificity. PC-3 cells were chosen because of their high tumorigenicity and invasiveness. After 7 days of cultivating C4-2b and PC-3 cells in RPMI 1640 as three-dimensional cultures embedded in Matrigel, cultures were examined by phase contrast and confocal microscopy. Untreated PC-3 cells grew as large cell masses and covered the surface of the Matrigel; whereas C4-2b cells formed large irregular clusters. After pretreatment with 5 μM of the F28 peptide, but not with L11 or control (nonrelevant peptide with similar amino acid combination) peptide, PC-3 cells formed smaller clusters and did not occupy the Matrigel surface. After the same treatment with F28 peptide, C4-2b cells formed regular spheroids. In some of them, an internal lumen could be observed by confocal microscopy (Fig. 4).

Identification of Cellular Binding Molecules for the Selected Phage/Peptides. We carried out the identification of putative cellular receptors for L11 and F28 peptides (selected as inhibitors of attachment of C4-2b cells to BMECs). We exploited a cDNA expression library constructed in T7 phage using mRNA purified from C4-2b cells for the identification of C4-2b cell surface-associated molecules that specifically bind selected phage and peptides. The cDNAs were inserted into the phage-based cloning vector T7Select-10b to generate a library of gene fusions of cDNAs with the phage capsid protein 10b. The constructed library contained ~1 × 10^9 independent clones as confirmed by plaque sequencing. The library was amplified once. Ten clones randomly selected for PCR evaluation revealed an average insert size of 1700 bp.

Biopanning was performed using affinity column chromatography. Two peptides with profound inhibitory activity (L11, F28) were immobilized on HiTrap columns. Twenty phage for each target from the last round of biopanning were individually amplified and sequenced. PCR products differed in size from 0.6 to 2.5 kb. Sequences were translated into amino acid code and analyzed. Sequenced products that were in frame with 10b capsid protein had a maximal size of ~200 bp. The sequences obtained from the selected clones were used to search GenBank databases. Identified matches were divided into range of 10 nm-10 μM. Six of the synthesized peptides inhibited attachment at 100 nm concentration by 30–40% (Fig. 3). This effect was dose dependent (data not shown). The blocking ability of the selected peptides was most efficient for C4-2b and C4-2 cells. Some inhibition was also observed for DU-145 and BPH cells; whereas PC-3 cell attachment was unaffected. When peptides were used in combination, inhibition efficiency was enhanced (Fig. 3).

Modulation by the Selected Peptides of Proliferation, Apoptosis, Motility, and Invasion of PC Cells. In addition to the inhibition of the attachment of PC cells to BMECs, we assayed several other potential activities of the selected peptides. This included evaluation of the ability of the selected peptides to alter the morphology of cell clusters in three-dimensional culture. C4-2b and PC-3 cells were maintained for 7 days in RPMI 1640 as three-dimensional cultures embedded in Matrigel. Peptides selected as inhibitors of C4-2b-cell attachment to bone marrow endothelial cells (BMECs) were added three times during the growth of the three-dimensional cultures at a concentration of 5 μM. After 7 days, cultures were examined by phase and confocal microscopy.
three categories as described in “Materials and Methods.” Results from the search are summarized in Table 2. Among the individual phage clones from the selected pool, one avidly bound to immobilized peptide L11. Sequence analysis showed that this clone, number 28, encodes a peptide that is homologous to the COOH-terminal region of the secreted protease, PSA. The identity of the phage fragment with the COOH-terminal end of the PSA sequence is 99%.

Query: 4 GSGSRTPSWPTPEQ 17 sequence of the insert clone 28 (GSGSRTPSWPTPE+); Sbjct: 792 GSGSRTPSWPTPEH 833, COOH termini of PSA. To confirm the binding specificity of the selected T7 phage to the target peptides, two types of assays described in the “Materials and Methods” have been performed. The phage coding the COOH-terminal part of PSA adhered to L11 twenty times stronger than nonrelated phage from the primary library. The binding of the PSA-coding phage number 28 to L11 decreased approximately four to six times when the phage was preincubated with L11 peptide or/and anti-PSA antibodies. Control antibodies or nonrelated peptides had no effect on the binding of phage number 28 to L11 peptide. Similar results were obtained with both assays.

**Selected Peptide Binds PSA.** We performed ELISA type assay to show that peptide L11, in addition to the interaction with the selected T7 phage 28, can also interact with PSA in vitro. The affinity of PSA to immobilized L11 peptide was higher by 49 and 46%, respectively, than to F23 and F28 peptides. Adhesion to L11 was also greater by 25% than to the L19 peptide, which, as L11, expressed a DPR sequence (Fig. 5; Table 1). A

**PSA as Cell–Cell Interacting Molecule.** To examine the possibility that PSA may be directly involved in the intercellular interaction between PC and BMECs, we used a cell–cell adhesion inhibition assay. To confirm the production of PSA by C4-2B cells, we performed immunofluorescent staining and Western blotting (Fig. 6). Immunofluorescent staining showed localization of PSA not only in secretory vesicles but also in cell protrusions (Fig. 6B). Pretreatment of C4-2B cells with anti-PSA antibodies diminished adhesion of PC to BMECs by 40% (Fig. 7). To further examine the possibility that PSA-binding sites are present on the surface of BMECs, we preincubated BMECs with various concentration of enzymatically active exogenous PSA (1 ng-50 μg). Preincubation of BMECs with 10 ng/ml PSA for 20 min attenuated adhesion of PC cells to BMECs by 30% (Fig. 7).

**Attenuation of PSA Expression in C4-2B Cells by RNAi Strategy.** Alters Adhesion to BMECs. We used an RNAi method to target PSA constitutively expressed in the C4-2B cell line. The constructs we designed encoded an RNA that targets the PSA mRNA transient transfection of C4-2B cells with PSA-specific siRNA-reduced PSA production in these cells (Fig. 6B). Adhesion of cells with altered PSA production to BMEC monolayer was attenuated by 30% (Fig. 7). Additional Candidates Identified by the Similarity to Sequences in Databases. In addition to PSA, we also identified candidates with a very high similarity to database sequences (100% identity, 21 translated amino acids matched). Some of these matches are displayed in Table 2. This included several ESTs from cDNA libraries.

### Table 2. T7 phage display library clones matching with sequences in databases

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gen Bank accession no.</th>
<th>Similarity</th>
<th>Matched amino acids</th>
<th>E-value*</th>
<th>Identity (%)</th>
</tr>
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<tbody>
<tr>
<td>P62L11</td>
<td>BE088394</td>
<td>Breast CDNA transl</td>
<td>37/52</td>
<td>3e-12</td>
<td>71</td>
</tr>
<tr>
<td>P62L11</td>
<td>P96100</td>
<td>Perlecan (not in frame)</td>
<td>16/31</td>
<td>3.3</td>
<td>50</td>
</tr>
<tr>
<td>P62L11</td>
<td>BE150559</td>
<td>Head-neck CDNA transl</td>
<td>37/52</td>
<td>6e-12</td>
<td>71</td>
</tr>
<tr>
<td>P62L11</td>
<td>BE740559</td>
<td>Ovary cDNA transl</td>
<td>22/24</td>
<td>1e-08</td>
<td>91</td>
</tr>
<tr>
<td>P69L11</td>
<td>BD071292</td>
<td>Secreted EST transl (patent)</td>
<td>21/21</td>
<td>2e-12</td>
<td>100</td>
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<tr>
<td>P63L11</td>
<td>U17040</td>
<td>PSA in frame</td>
<td>14/14</td>
<td>4e-04</td>
<td>99</td>
</tr>
<tr>
<td>P67L11</td>
<td>BM75625</td>
<td>Stomach and other libraries CDNA (transl)</td>
<td>19/19</td>
<td>0.002</td>
<td>100</td>
</tr>
</tbody>
</table>

*transl: translated; EST, expressed sequence tag; PSA, prostate-specific antigen. E-value, expectation value.
Treatment of C4-2b or BMEC

Fig. 7. Inhibition of the attachment of prostate cancer (PC) C4-2b cells to bone marrow endothelial cells (BMECs) by anti-prostate-specific antigens (anti-PSA) polyclonal antibodies and by PSA. Diminished attachment of C4-2b cells when PSA production was inhibited. Cell–cell adhesion assay has been performed as described in the legend to Fig. 2. Control antibodies, nonrelated IgG anti-PSA-polyclonal antibody from Dako Corp (dilution 1:8000). C4-2b PSA siRNA-cells transiently transfected with PSA-specific siRNA. C4-2b GAPDH siRNA-control, cells transiently transfected with GAPDH-specific siRNA. Adhesion was assayed in quadruplicate, and the experiments were performed at least three times. * P < 0.05.

prepared from solid tumors or from malignant cultured cells. We also identified a similarity to amino acid sequences of several proteins in one of the translational frames (Table 2, perlecan). The selected candidates will be further characterized, and their function will be assayed.

DISCUSSION

The choice of the primary target cells for biopanning with random peptide phage libraries was dictated by the following rationale. The LNCaP cell-derived series of human PC cell lines closely mimic clinical PC and are a superb tool for studying PC in vitro and in vivo. LNCaP cells are able to produce tumors when inoculated s.c. into an intact (not castrated) host, but do not produce experimental metastasis in animals (15). Experimental manipulation involving cellular interactions between LNCaP and osteosarcoma-derived osteoblasts allowed the establishment of androgen-independent C4-2, and C4-2B cell lines, which are capable of metastasizing to lymph nodes and bones (16). In contrast to other PC cell models (PC-3), C4-2B cells form osteoblastic but not osteolytic bone metastases in experimental animals. These tumors produce PSA when they are maintained as tumor xenografts in vivo (17).

Peptides synthesized based on the selection from phage-displayed libraries inhibited C4-2b adhesion to BMECs up to 40% at 100 nm concentration. The inhibitory activity of these peptides may be further improved by the affinity maturation process. The selected peptides altered attachment of other PC cells not as effectively as C4-2b, and adhesion of PC-3 cells was not affected. Cell type specificity of the identified phage is not surprising. In our previous study we identified phage pg35 that was able to block the spreading of the LNCaP cells used for the selection and their close relatives (C4-2b and C4-2 cell lines) but was not effective against PC-3 and DU-145 cells or against several other cells of nonprostate origin (11). C4-2b and PC-3 cells, two cell types that were used for studying bone metastasis in experimental animals, display many biochemical and functional differences. This includes different expression and/or altered conformation of secreted and cell surface molecules including cell adhesion molecules. All of the assayed peptides were not able to inhibit the attachment of PC-3 cells. For peptide L11, it is established that it interacts with PSA. However, peptides F28 and F23 display a very weak interaction with PSA. Because PC-3 cells do not express PSA, this may explain the ineffectiveness of L11 against this cell type. F28 peptide might share the same or similar binding sites on the surfaces of both cell types. However, mechanism of PC-3 and C4-2b cells interactions with BMECs might be quite different. Because cell–cell adhesion is a cooperative process, PC-3 cells might use more types of cell adhesion molecules simultaneously. Therefore, inhibition of most cell adhesion molecules participating in the interactions of PC-3 cells with BMEC by the selected peptides may not be effective enough to substantially block PC-3-BMEC interactions.

Among the characteristic features of epithelial tumors is the loss of cellular differentiation and proliferation control and, as a result, alterations of epithelial tissue architecture. Tissue function is maintained by a dynamic interplay between epithelial cells and their microenvironment. These interactions involve insoluble extracellular matrix, stroma consisting of fibroblast, adipose, vasculature, and resident immune cells as well as the conventional milieu of cytokines and growth factors (18). As a result of an alteration in these interactions, changes in the tissue organization are observed in many epithelial cancers including invasive prostatic carcinomas (19). The ability to form acinar structure was shown for low tumorigenic breast epithelial cells, whereas more malignant cells grew as chaotic clusters. Furthermore, anti-β1 blocking antibodies were able to revert the morphology of these clusters to acinar type (14). The identification of other factors influencing epithelial tumor tissue architecture will be helpful in understanding and controlling the process of tissue structuring. Therefore, we assayed selected peptides for the ability to revert the morphology of three-dimensional clusters to acinar phenotype. One of the assayed peptides (F28) alters the morphology of three-dimensional clusters. This peptide, as well as peptide L11, was chosen for this assay because of its ability to alter C4-2b adhesion to BMECs. The peptide F28 was effective against both types of the examined cells (C4-2b and PC-3), despite altering the adhesion of only C4-2b cells to BMEC. The mechanism of tissue architecture regulation is not well studied yet. F28 peptide may share the same epitopes on the surfaces of both cell types (C4-2b and PC-3). Interactions of this peptide with cell surface binding site is effective in altering morphology of three-dimensional clusters formed by both cell types but not sufficient to attenuate an adhesion of PC-3 cells to BMECs.

Phage display is a powerful technique for the identification of peptides or proteins that bind to other molecules. Most applications of phage display systems have involved the selection of short peptides or naturally secreted proteins compatible with the requirement for translocation of the cloned proteins across the Escherichia coli inner membrane before incorporation into filamentous phage. Recent advances in the use of lytic bacteriophage vectors such as T7 have provided an alternative that is independent of the E. coli secretion machinery and its limitations. A variety of biologically active peptides and proteins have already been cloned using the T7 select system (20–22). Despite the limitations of the use of phage display systems for the expression of eukaryotic proteins related to the lack of eukaryotic posttranslational modification machinery in bacteria, this system has been successfully used for the identification of several eukaryotic membrane proteins in which these modifications are critical. Receptors for natural products (23), for phosphatidylinerine-specific clearance of apoptotic cells (24), and for endothelial surface protein aminopeptidase P (25) have been identified using T7-based cDNA
expression libraries. Therefore, we relied on this approach and have exploited it for the identification of molecules expressed on the surface of PC cells, which were binders for the peptides previously selected from random phage display library. We selected from the T7 phage displayed library the phage that codes the COOH-terminal part of the secreted protein PSA. This phage is identified in the biopanning of the L11-selected peptide.

Although PSA is a secreted protease, when intercellular localization of PSA has been studied by immunoelectron microscopy in addition to secretory granules, it is also present on the plasma membrane and in secretory blebs in malignant prostate cells (26). PSA is expressed in C4-2b cells. Therefore, peptide L11 could serve as a synthetic ligand for PSA that was associated with the surfaces of C4-2b cells. This is the biologically reasonable match with high similarity to the native protein.

Because L11 was able to block interactions of C4-2b cells with BMECs and interacts with PSA, it is possible that PSA may be directly involved in the interaction of PC cells with BMECs. To test this hypothesis, we performed cell–cell adhesion experiments using anti-PSA antibodies as potential inhibitors of adhesion. To investigate the role of PSA produced by PC cells in the interaction of these cells with bone marrow endothelium, we knocked out PSA in C4-2b cells using siRNA technology. Cells with reduced production of PSA revealed low adhesive ability. This provides additional evidence of the direct involvement of PSA in PC cells interaction with bone marrow endothelium.

In conclusion, we propose that PSA exposed on the surface of C4-2B cells might interact directly or indirectly with some yet non-identified molecules on the surface of BMECs. Although PSA is widely used as a serum marker to screen for PC, a pathophysiological role is uncertain (27). Novel biological properties of PSA including its antiangiogenic activity have been reported (28). These observations implicate the presence of receptors or substrates for PSA on the surface of endothelial cells. Therefore, PSA may be the molecule produced by metastatic PC cells that participates in direct interactions of these cells with BMECs. This interaction is likely not universal because not all PC cells metastasizing to bone express PSA, but it probably provides a supportive mechanism, which must be further studied.

In addition, the approach provided herein allows an identification of cell–cell adhesion molecules.

ACKNOWLEDGMENTS

We thank Drs. John Kovach and Dmitry Gnatenko for valuable advice, fruitful discussion, and support. We are also grateful to Dr. K. Malinowski for manuscript reading and helpful suggestions.

REFERENCES

Prostate Cancer Cell Adhesion to Bone Marrow Endothelium: The Role of Prostate-Specific Antigen


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