Preferential Adhesion of Prostate Cancer Cells to Bone Is Mediated by Binding to Bone Marrow Endothelial Cells as Compared to Extracellular Matrix Components in Vitro

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INTRODUCTION
Circulating tumor cells attach to specific CAMs expressed on the luminal surface of endothelial cells lining the organ microvasculature (1–3). The surface protein expression of endothelial cells derived from various organ microvasculatures is determined, in part, by the ECM of the organ. We investigated how prostate cancer cell adhesion to HBME cells is affected by growing HBME cells on selected organ-derived ECM proteins in vitro. Growth of HBME cells and immortalized human aortic endothelial cells on bone, kidney, and placenta ECM proteins significantly increased their ability to bind PC-3 cells. This increased adhesion was not dose dependent and was not demonstrated with human dermal microvascular endothelial cells. Scanning electron microscopic analysis demonstrated that prostate cancer cells adhered directly to the endothelial cells and not to the underlying substrata. These results suggest that unidentified cell adhesion molecules are expressed or up-regulated on the apical surfaces of human aortic endothelial cells and HBME cells grown on bone, kidney, and placenta ECMs. These results also strongly demonstrate that the adhesion of prostate cancer cells to bone may be initiated by direct binding to endothelial cells rather than direct binding to exposed ECM components.

ABSTRACT
We have demonstrated previously that the preferential adhesion of prostate cancer cells to human bone marrow endothelial (HBME) cells may contribute to their preferential metastasis to bone. Although a subject of debate, it has been postulated that the endothelial cells of the bone marrow are fenestrated. It is unknown therefore whether prostate cancer cells adhere preferentially to the extracellular matrix (ECM) or the endothelial cells. It has also been demonstrated in other organ systems that the types of cell adhesion molecules on the surface of endothelial cells lining the organ microvasculature are determined, in part, by the ECM of the organ. We investigated how prostate cancer cell adhesion to HBME cells is affected by growing HBME cells on selected organ-derived ECM proteins in vitro. Growth of HBME cells and immortalized human aortic endothelial cells on bone, kidney, and placenta ECM proteins significantly increased their ability to bind PC-3 cells. This increased adhesion was not dose dependent and was not demonstrated with human dermal microvascular endothelial cells. Scanning electron microscopic analysis demonstrated that prostate cancer cells adhered directly to the endothelial cells and not to the underlying substrata. These results suggest that unidentified cell adhesion molecules are expressed or up-regulated on the apical surfaces of human aortic endothelial cells and HBME cells grown on bone, kidney, and placenta ECMs. These results also strongly demonstrate that the adhesion of prostate cancer cells to bone may be initiated by direct binding to endothelial cells rather than direct binding to exposed ECM components.

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3 The abbreviations used are: CAM, cell adhesion molecule; HBME, human bone marrow endothelial; ECM, extracellular matrix; HDMVEC, human dermal microvascular endothelial cell; BAEC, bovine aorta endothelial cell; TAM, tumor attachment modulator; FBS, fetal bovine serum; HAEC, human aorta endothelial cell.
MATERIALS AND METHODS

Cell Lines. The prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). The immortalized HBME cell line was developed in our laboratory (13). HAECs were purchased from Clonetics (San Diego, CA) and immortalized with SV40 as described previously (13). The HDMVEC line was kindly provided by Dr. Alvin Schmair (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI). The PC-3 cell line was maintained in RPMI 1640 supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin. HBME and HAEC were grown in 89% DMEM, 10% FBS, and 1% penicillin/streptomycin. HDMVEC

Fig. 1. PC-3 cell adhesion to ECM components. Wells were coated with 0.1 μg/100 μl (0.1), 1 μg/100 μl (1), and 10 μg/100 μl (10) collagen I (C), laminin (L), fibronectin (F), and transferrin (T) with error bars. The control (●) is the magnitude of PC-3 cell adhesion to plastic. Statistically significant values relative to the control are indicated by * (P < 0.05). Bars represent 95% confidence intervals based on the ANOVA.

Fig. 2. PC-3 cell adhesion to ECM components at a concentration of 10 μg/ml versus a monolayer of HBME cells. The magnitude of PC-3 cell adhesion to HBME cells grown on plastic serves as the control. Statistically significant values relative to the negative control are indicated by * (P < 0.05). Bars represent 95% confidence intervals based on the ANOVA.
line was maintained in complete endothelial cell growth medium (Clonetics) supplemented with 10% FBS and 1% penicillin/streptomycin.

Organ Matrix Preparation. Human spinal bone fragments and kidney samples were obtained from a warm autopsy sample and processed as described previously to obtain ECM proteins (14). Briefly, bone and kidney samples were homogenized in cold 0.02M PBS [0.5 mM NaH₂PO₄, 1.9 mM Na₂HPO₄, and 17.9 mM NaCl (pH 7.3)] and allowed to stand for 5 min. The preparation was centrifuged at 2500 × g for 15 min, and the ECM-soluble protein-rich supernatants were recovered after two extractions of 5 min. This preparation was coated onto assay plates at various concentrations, incubated overnight at room temperature under sterile conditions, and stored at 4°C.

Adhesion Assays. Adhesion assays were performed as reported previously (13). Briefly, snap-apart 96-well tissue culture plates (Fisher Scientific, Pittsburgh, PA) were coated with crude bone and kidney matrices. Plates were incubated overnight at room temperature under sterile conditions and stored at 4°C until needed. Assay plates were also coated with ECM components (i.e., human collagen I, human fibronectin, and mouse laminin I) and human transferrin at various concentrations according to the manufacturer’s instruction (Collaborative Biomedical Products, Bedford, MA). Endothelial cells were seeded onto various substrata at a concentration of 900 cells/μl and grown to confluence. Tumor cells were removed from the flask by a 15–20-min treatment with 0.5 mM EDTA in HBSS. Once the EDTA solution was removed, the cells were resuspended in adhesion media and layered on a confluent layer of endothelial cells for 30 min at 37°C. In addition, radiolabeled tumor cells were applied to placenta matrix, crude bone and kidney matrices, various ECM components, and transferrin. Again, plates were washed three times in PBS, and adhesion was determined by counting individual wells on a gamma counter. Cell adhesion was reported relative to the adhesion of controls, which were set to 100.

Scanning Electron Microscopy. For high resolution of prostate cancer cell adhesion to HBME monolayers, scanning electron microscopy was done. Adhesion assays were done as described above without radiolabeling the cancer cells. HBME monolayers and attached tumor cells were fixed in 2.5% glutaraldehyde for 1 h at room temperature and then placed in a refrigerator overnight. The monolayers were rinsed three times in 0.025 M cacodylate buffer (pH 7.3) and subsequently post-fixed in 1% osmium tetroxide for 1 h at room temperature. Dehydration was accomplished by a 10–15-min exposure to ethanol in the following ascending order: (a) 30%; (b) 50%; (c) 70%; (d) 95%; and (e) 100%. The samples were exposed to the 95% and 100% solutions twice. The alcohol was displaced via three 20-min changes of hexamethyldisilazane. Residual hexamethyldisilazane was evaporated overnight in a fume hood before mounting samples on specimen mounts for light sputter coating.

Statistical Analysis. Two-factor ANOVA was applied to log-transformed radioactive decay counts for each experiment. The two factors included were experimental group and batch. The estimates represent the expected values of the experimental group relative to the control group. Tukey’s multiple comparisons were used to verify the result for the ANOVA. In the graphs, the control group is plotted at 100. The estimated value

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**Fig. 3** HBME cells grown on ECM components. HBME cells were grown on collagen I (C; △), laminin (L; ■), fibronectin (F; ○), transferrin (T; ●) with error bars, and plastic (P; □) substrata at the same concentrations described in Fig. 1. The control is the magnitude of PC-3 cell adhesion to HBME cells grown plastic. Statistically significant values relative to the control are indicated by * (P < 0.05). Bars represent 95% confidence intervals based on the ANOVA.
RESULTS

Endothelial cells lining the blood vessels in red bone marrow have been reported to be fenestrated (11, 12). Circulating tumor cells could potentially enter the bone microenvironment via these fenestrae once they have attached to specific CAMs on bone marrow endothelial cells or attached directly to a specific ECM component, for example fibronectin. Therefore, we compared the binding of PC-3 cells to a variety of bone ECM components (fibronectin, laminin, collagen I, and transferrin). The data demonstrated that PC-3 preferentially adhered to all ECM components except transferrin (Fig. 1). The adhesion was dose dependent for collagen I and laminin I. At the highest dose, PC-3 cells preferentially adhered to laminin over collagen, fibronectin, and a monolayer of HBME cells (Fig. 2). Although there was no difference between PC-3 cell adhesion to collagen I and laminin I. At the highest dose, PC-3 cells preferentially adhered to laminin over collagen, fibronectin, and a monolayer of HBME cells, there was a preference for HBME cell monolayers over fibronectin. We next investigated whether the above-mentioned ECM components altered the binding of prostate cancer cells to HBME by growing HBME cells on individual ECM components. There was a decrease in PC-3 cell adhesion to HBME grown on laminin at 0.1 μg/100 μl, on fibronectin at 10 μg/100 μl, and on transferrin at 1 μg/100 μl and 10 μg/100 μl compared with HBME cells grown on plastic (control) and other typical ECM components (Fig. 3).

To examine the net effect the various ECM proteins have on PC-3 adhesion to various organ-derived ECM proteins, adhesion assays were performed using increasing concentrations of bone, kidney, and placenta ECM proteins. Kidney and placenta ECM proteins were used as negative controls because prostate cancer cells rarely metastasize to the former and never metastasize to the latter. The data demonstrated that PC-3 cells adhered more to HBME monolayers than to bone and kidney ECM proteins at all concentrations (Fig. 4). Only the highest concentration (i.e., 10 μg/100 μl) of placenta ECM had a comparable affinity for PC-3 (Fig. 4). PC-3 cell adhesion was dose dependent on bone and placenta ECM proteins.

Pauli and Lee (5) demonstrated that large aorta endothelial cells could be modulated to express organ-specific adhesion molecules by growing these endothelial cells on organ-processed ECMs. Both HAEC and HBME cells were grown to confluence on bone, kidney, and placenta ECM proteins to determine their affect on PC-3 cell adhesion to HBME cells and HAECs. Growth of HBME cells and HAECs on the selected organ’s ECM proteins significantly increased their affinity for PC-3 cells (Fig. 5, A and B). This increased affinity was not dose dependent. Because the response was not dose dependent, we used the highest concentration (10 μg/100 μl) to examine the net effect of the various matrix proteins on the ability of the three endothelial cell types to bind PC-3 cells. The data demonstrated that PC-3 preferentially adhered to HBME cell monolayers as compared with HAECs and HDMVECs (Fig. 6A). However, the growth of HDMVECs on the selected organ matrices did not alter their ability to bind PC-3 (Fig. 6B). An increased adhesion was demonstrated for bone ECM-modulated HBME cells and HAECs. An increased adhesion was also demonstrated for kidney and placenta ECM-modulated HAECs. To our surprise, kidney and placenta matrices failed to enhanced adhesion of PC-3 cells to HBME in this experiment as it did in the previous experiment (Fig. 5A) and in other experiments (data not shown). The reasons for this outcome are not known. However, despite this outcome, the data strongly support the expression or up-regulation of CAMs on the endothelial cells primarily because PC-3 cell adhesion to exposed organ matrices, particularly bone and placenta, was dose dependent. However, PC-3 cell adhesion to HBME cells and HAECs modulated by bone

Fig. 4 PC-3 cell adhesion to kidney, bone, and placenta ECM proteins. Wells were coated with 0.1 μg/100 μl (0.1, 1 μg/100 μl (1), and 10 μg/100 μl (10) of kidney (K; containing *), bone (B; □), and placenta (P; ▪) matrices. The magnitude of PC-3 cell adhesion to plastic (●) and monolayers of HBME cells (○ with error bar) serves as a negative and positive control, respectively. Statistically significant values relative to the positive control are indicated by * (P < 0.05). Statistically significant values relative to the positive control are indicated by ** (P < 0.05). Values that are significantly different from both negative and positive controls are indicated by X (P < 0.05). Bars represent 95% confidence intervals based on the ANOVA.
and placenta matrices was not dose dependent, as one would expect if the prostate cancer cells were binding to both exposed matrix components and endothelial cells or exposed matrix proteins over the endothelial cells. For instance, the relative magnitude for PC-3 cells adhering to HBME monolayers grown on bone matrix at increasing concentrations was relatively the same as that for control HBME monolayers (Fig. 5A). This was not the case for PC-3 cells adhering to naked bone matrix (i.e., no endothelial cells were seeded on the matrix). There was a significant increase in the magnitude of PC-3 cell adhesion, correlating with increasing concentrations of ECM proteins.

To confirm that PC-3 cells were adhering to the endothelial cells and not to exposed ECM proteins, scanning electron microscopy was performed after adhesion assays under control conditions (Fig. 7). The data demonstrated that more PC-3 cells bind HBME cells growing on bone matrix compared with plas-
tic (i.e., control; Fig. 7, C and D). The data also confirm that PC-3 cells were binding the apical surfaces of the endothelial cells and not the exposed underlying bone matrix.

DISCUSSION

The metastasis of prostate cancer cells to the skeleton is well described by the “seed and soil” hypothesis (15, 16). Studies have shown that prostate cancer cells grow well in the bone marrow (17). Lehr and Pienta (13) suggested that prostate cancer cells selectively colonized bone because of their preferential adhesion to bone marrow endothelial cells. The initial colonization of secondary organs, however, is mediated by attachment of tumor cells to microvascular endothelium (18, 19). In the present study, we attempted to determine the role of organ-derived ECM proteins on prostate cancer cell adhesion to endothelial cells.

Fig. 6 PC-3 cell adhesion to three distinct endothelial monolayers (A) and their growth on the ECM proteins of selected organs at 10 μg/μl (B). A, the magnitude of PC-3 cell adhesion to HDMVEC monolayers serves as a control. Statistically significant values relative to the controls are indicated by * (P < 0.05). The relative magnitude of PC-3 cell adhesion to HBME is significant compared to the magnitude of PC-3 cell adhesion to control endothelial monolayers and HAEC monolayers, and this is indicated by ** (P < 0.05). B, the magnitude of PC-3 cell adhesion to each endothelial cell type grown on plastic serves as a control and is represented by ●. ●, endothelial cells grown on bone ECM proteins; ■ containing *, endothelial cell grown on kidney ECM proteins; ▲, endothelial cells grown on placenta ECM proteins. Statistically significant values relative to the controls are indicated by * (P < 0.05). Bars represent 95% confidence intervals based on the ANOVA.
Fenestrae are common in the bone marrow endothelial cell lining and are probable sites of vascular permeability. Circulating blood cells use fenestrae to exit the blood circulation. It is therefore conceivable that bone-metastasizing cancer cells could also use fenestrae to gain access to the bone marrow microenvironment by attaching to CAMs in the ECM such as laminin (12). Our study demonstrated preferential adhesion of PC-3 cells to HBME cells over fibronectin and comparable adhesion of PC-3 cells to HBME cells and collagen I. Only the highest concentration of laminin exceeded HBME cells’ affinity for PC-3 cells. Although transferrin is mitogenic to prostate cancer cells and is prevalent in the bone microenvironment, it may not play a significant role in the adhesion of prostate cancer cells to bone marrow endothelium (20).

Because bone matrix was expected to enhance PC-3 cell adhesion to HBME cells, the enhanced adhesion of PC-3 to HBME cells grown on kidney and placenta matrices was unexpected. Pauli and Lee (5) showed that tumors that metastasized to a specific organ attached more to endothelial cells grown on ECM derived from that specific organ than to endothelial cells grown on ECM derived from other organs. For instance, lung metastatic tumor cells adhere preferentially to BAECs modulated by lung-derived TAMs versus BAECs modulated by liver-derived TAMs. Our results are not consistent with this observation. One explanation maybe that the endothelial cells used in Pauli and Lee’s investigation (5) were derived from the aorta, whereas ours were derived from microvessels in the bone marrow. Secondly, our HBME cells were transformed by SV40 large T antigen. Nevertheless, these results suggest that adhesion molecules expressed on HBME cells are up-regulated by bone, kidney, and placenta matrices. These results actually support the “soil hypothesis.” This may further demonstrate that prostate cancer cells can adhere to endothelial cell modulated by kidney matrix, but growth is limited in the kidney tissue because it lacks the required growth factors (17, 20).

Although there was no difference between PC-3 cell adhesion to HBME cells modulated by bone, kidney, or placenta matrix, there was a significant difference between the adhesion of PC-3 cells directly to bone and kidney matrices. PC-3 cells adhered more to the bone matrix than to kidney matrix at higher concentrations. PC-3 cells adhere well to collagen I and laminin, but not to fibronectin (Fig. 8; Ref. 21). The preferential attach-
ment of PC-3 cells to bone matrix over kidney matrix may be mediated by the increased amounts of laminin present in bone marrow extracts enriched for soluble ECM proteins (14).

In summary, our data demonstrate that bone, kidney, and placenta matrices significantly increased PC-3 cell adhesion to HBME cells by either up-regulating or inducing the expression of unidentified CAMs. None of ECM proteins (fibronectin, laminin I, and collagen I) found in bone are solely responsible for this enhanced adhesion. This effect may be mediated by the net effect of all of the proteins and other components in the organ matrix. The enhanced adhesion of PC-3 cells to monolayers of HBME cells grown on bone matrix suggests that binding in vitro is not a result of unseen gaps that would therefore allow PC-3 cells to bind preferentially to exposed ECM components such as laminin. Future investigations will continue to explore other methods of identifying CAMs involved in the interaction of prostate cancer cells and HBME cells in vitro and in vivo and how their expression is regulated. Once specific CAMs are identified, they can be studied, and sequences that are important for cell adhesion can be targeted in an attempt to prevent bone metastasis in prostate cancer patients. Such a strategy was successful using a Pro-His-Ser-Cys-Asw (PHSCN) sequence to inhibit PHSRN-mediated invasion and metastasis of MATLyLu in a rat model system. PHSRN is a peptide sequence of plasma fibronectin (22).

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