

CD44 Potentiates the Adherence of Metastatic Prostate and Breast Cancer Cells to Bone Marrow Endothelial Cells

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ABSTRACT

The aim of this current study was to examine the significance of CD44 expression in mediating cancer cell adhesion to human bone marrow endothelial cell(s) (hBMEC). Differential CD44 expression on two metastatic prostate cancer cell lines, PC3 (CD44 +ve) and DU145 (CD44 –ve) and four breast cancer cell lines was confirmed by immunoblotting and immunocytochemistry. In cell adhesion assays, PC3 but not DU145 cells demonstrated a rapid adhesion to hBMECs. Treatment of PC3 cells with a neutralizing antibody against CD44 standard (CD44s) and CD44 splice variants decreased PC3 cell adhesion to hBMECs. Similarly, depletion of CD44 expression using RNA interference decreased the ability of PC3 cells and two CD44 +ve breast cancer cell lines (MDA-MB-231 and MDA-MB-157) to bind FITC-conjugated hyaluronan (FITC-HA) and to adhere to hBMECs. In contrast, transfection of DU145 cells or the T47D and MCF-7 breast cancer cell lines to express CD44s increased cell surface binding of FITC-HA and cell adherence to hBMECs. Treatment of PC3 and MDA-MB-231 cells but not hBMECs with hyaluronidase attenuated cell adhesion, suggesting that cell surface expression of CD44 on prostate and breast cancer cells may promote the retention of a HA coat that facilitates their initial arrest on bone marrow endothelium.

INTRODUCTION

Metastatic prostate cancer and breast cancer are each associated with an increased risk and incidence of skeletal micrometastasis. Metastasis to the bone is thought to result from the ability of these cancer cells to arrest on, adhere to, and extravasate across the bone marrow endothelium into the underlying bone matrix (1). Under normal physiology, the bone marrow endothelium is a dynamic cell interface permitting the egress of mature myeloid and lymphoid cells into the circulation and facilitating uptake of circulating progenitor cells (2, 3, 4). The mechanism underpinning the selective adherence of prostate and breast cancer cells is poorly understood but has been proposed to mirror those mechanisms that facilitate the trafficking of homing lymphocytes and progenitor cells into the bone marrow.

The adhesion of lymphocytes to endothelium is proposed to follow a docking and locking mechanism, underpinned by a specific and sequential activation of cell surface adhesion receptors of the selectin- and integrin-receptor families (5). Studies investigating the mechanism that promotes the selective adhesion of prostate cancer cells to bone marrow endothelium have primarily focused on the role of integrin receptors in mediating the “firm” or secondary adhesion (6, 7, 8). Recent studies have also implicated hyaluronan (HA) in promoting adhesion to bone marrow endothelium (9, 10). HA is a polymeric glycosaminoglycan with ubiquitous expression that is essential for growth and motility of normal and neoplastic cells (11, 12, 13). The adhesion of prostate cancer cells to bone marrow endothelial cells was

shown to correlate with their ability to maintain a pericellular distribution of HA. Specifically, the PC3 cell (coincidentally derived from a prostate cancer metastasis to the bone) adhered strongly to bone marrow endothelial cell(s) (BMEC), but the LNCaP and DU145 cell lines, both of which failed to exhibit a pericellular HA distribution, demonstrated weak adherence. Maximal adhesion of PC3 cells to BMECs and retention of HA on the surface of this cell was associated with the elevated synthesis of HA and the overexpression of HA synthase. However, the mechanism by which PC3 cells retain HA on their surface is still unknown. Pericellular HA retention is not unique to cancer cells. An intriguing parallel may be drawn to chondrocytes that upon detachment from neighboring cells rapidly respond to produce a pericellular HA-coat (14), the maintenance of which is dependent on the expression and function of the HA-receptor CD44.

CD44 is the principal receptor of the hyaladherin-receptor family. Alternative splice variants give rise to a number of CD44 isoforms, but each variant retains the ability to bind HA on the conserved ecto-domain of the receptor (15). Multiple functions and cellular responses have been attributed to the activation of CD44, including the induction of cell motility, activation of cell survival responses, and the promotion of cell adhesion. Indeed, interactions between HA and CD44 have been shown to underpin the initial arrest of lymphocytes to vascular endothelial cells (16, 17) and are required for the inflammation-promoted extravasation of superantigen-stimulated T cells into the peritoneal cavity *in vivo* (18). Furthermore, HA/CD44 interactions have also been implicated in promoting the homing and transendothelial invasion of myeloma cells across the bone marrow endothelium (19). The significance of CD44 function in promoting colonization of the bone by solid tumors is only beginning to emerge. Recently, hepatocyte growth factor-promoted adhesion of breast cancer cells to bone marrow endothelium was shown to correlate with induction of CD44 expression (20).

We have examined the significance of CD44 expression in mediating the adhesion of prostate cancer and breast cancer cells to BMECs. Adhesion of prostate and breast cancer cells to these endothelial cells was directly correlated with CD44 expression on the cancer cell. The proposed function of CD44 is to promote HA retention on the cancer cell thereby facilitating presentation of a cancer cell-associated HA matrix to activated CD44 receptors expressed on the bone marrow endothelium.

MATERIALS AND METHODS

Cell Culture and Reagents. PC3 and DU145 human prostate carcinoma cell lines and MDA-MB-231, MDA-MB-157, T47D, and MCF-7 breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in manufacturer's recommended media (DMEM or RPMI 1640) containing 10% FCS and 2% L-glutamine (Life Technologies, Inc. Paisley, United Kingdom). The human bone marrow endothelial cell line (hBMEC) was kindly provided by Dr. Babette B. Weksler (Weill Medical College of Cornell University, New York, NY) and was maintained in DMEM supplemented in 5% FCS, 10 mM HEPES, 3 mM L-glutamine and Pen/Strep. All cells were grown to 70% confluency for experimentation. Chemicals were supplied by Sigma (St. Louis, MO) unless otherwise stated.

Plasmid Construction. Full-length human CD44s cDNA was sub-cloned from mRNA extracted from PC3 cells by reverse transcription-PCR. The

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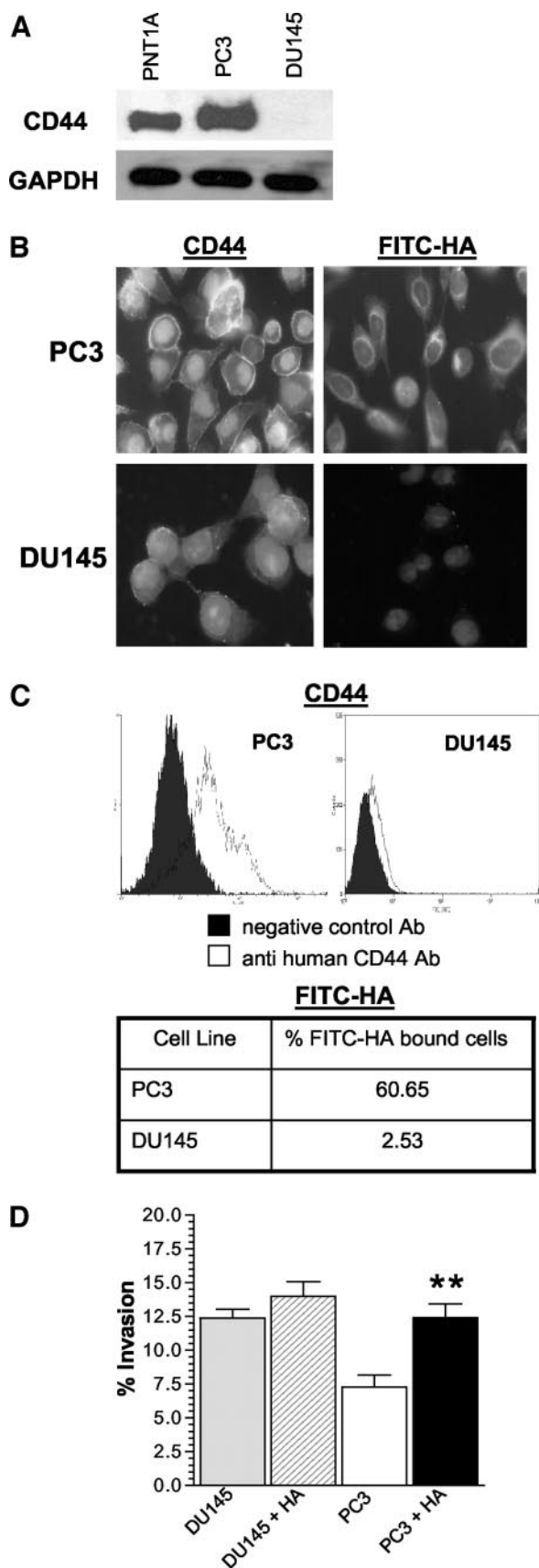


Fig. 1. Characterization of differential CD44 expression on metastatic prostate cancer cell lines. *A*, expression of CD44 on PNT1-A and PC3 but not DU145 cells was confirmed by immunoblotting conducted on protein lysates extracted from these cell lines. *B*, the differential expression of CD44 and the resultant capacity to bind FITC-HA by PC3 and DU145 cells was demonstrated by immunocytochemistry (magnification $\times 400$). *C*, expression of CD44 (*top panel*) and binding of FITC-HA (*table, bottom panel*) was detected

forward and reverse primers incorporated an *EcoRI*/Kozak sequence and *XbaI* restriction sites, respectively, as shown: CD44 *EcoRI* forward, CGAATTCGCGCCATGGACAAGTTTTGGTGGCAGCA, and CD44 (*XbaI*) reverse, GGTCCACGTCTCTAGACGGCCGACCCCAATCTTCAT. The amplified CD44s cDNA sequence was A-tailed, phenol-chloroform extracted and inserted into the pcDNA 3.1/v5 His TOPO vector (Invitrogen, Paisley, United Kingdom). Insertion of the plasmid in the sense direction was confirmed using *XbaI* and *HindIII* restriction digests, and validation of the correct CD44 coding sequence was confirmed by automated sequence analysis.

Transfection. DU145 cells and MCF-7 cells were transfected using GeneJuice (Novagen, Madison, WI) whereas T47D cells were transfected using Lipofectamine (Invitrogen) according to manufacturer's guidelines. Post-transfection, cells were harvested and the transfection efficiency determined by immunocytochemical and biochemical detection of CD44H expression using antihuman CD44 antibody (R&D Systems, Abingdon, United Kingdom).

RNA interference (RNAi)-mediated Depletion of CD44 Expression in PC3 Cells. Two oligonucleotides corresponding to nucleotide sequences in exon 3 of CD44 were synthesized commercially (Si1, 5-AACUCCAUCUGUGCAGCAAAC-3' and Si2, 5-AACACCUCCAGUAUGACACA-3'). A scrambled oligonucleotide of sequence (5'-AAAACCGACGGCUAUCUCUCA-3') was also used in these experiments. PC3 cells (40% confluency) were washed twice in sterile PBS and then incubated with a transfection mixture comprising OPTIMEM1, Oligofectamine (Invitrogen), and the CD44-directed oligonucleotides (final concentration range 100–150 nM) for 4 h at 37°C, after which cells were replenished with 20% v/v FCS-enriched RPMI 1640. Transfection was repeated after 24 h to effect greater suppression of CD44 expression. Transfected cells were incubated at 37°C for a further 48 h before harvesting for experimentation. Depletion of CD44-receptor expression in transfected cells was confirmed by immunocytochemistry and immunoblotting.

CD44 and Hyaluronan Immunocytochemistry. Cells were grown to 70% confluency on 22 \times 22 mm microscope coverslips, washed with PBS, then incubated for 1 h at room temperature with an antihuman CD44 monoclonal antibody (R&D Systems) at a final dilution of 1:200. In certain experiments to quantify intracellular localization of CD44, human umbilical vein endothelial cell(s) (HUVEC) were fixed with acetone before addition of the primary antibody. Otherwise, experiments were conducted to detect cell surface expression of CD44 on live cultured cells. Primary antibody was removed by repetitive washes with PBS after which antimouse FITC secondary antibody was added for 1 h at room temperature. Cells were washed in PBS and nuclei counterstained with propidium iodide. FITC-hyaluronic acid (FITC-HA, kindly provided by Prof. Clare Isacke, Institute of Cancer Research, London) was used to confirm functional activity of CD44H-HA binding ability. Cells were grown as before but treated with FITC-HA 1:100 at room temperature for 1 h to detect cell surface binding, then washed in PBS and treated with propidium iodide before examination by fluorescence microscopy.

Flow Cytometry. Cells were trypsinized, resuspended in serum-free medium without phenol red at a density of 5×10^5 cells/ml, and incubated with mouse antihuman CD44 monoclonal antibody (R&D systems) for 1 h at 4°C. After three washes in serum-free medium, cells were incubated for 1 h at 4°C with a rabbit antimouse IgG FITC-conjugated Ab (1:40 dilution, Dako, Ely, United Kingdom). Cells were washed twice in serum-free medium media, resuspended in PBS and 0.05% BSA, and analyzed for fluorescence on the flow cytometer (Becton Dickinson, Oxford, United Kingdom). To determine FITC-HA binding to cells, 1×10^6 cells were washed twice in HBSS containing 2.5% (v/v) FCS, resuspended in 100 μ l of FITC-HA (diluted 1:100 in HBSS), and incubated for 1 h at 4°C. Cells were washed twice with HBSS containing 2.5% (v/v) FCS, resuspended in 500 μ l of a 1% (v/v) paraformaldehyde solution in PBS, and analyzed on the flow cytometer.

Western Blotting. Cells were harvested, precipitated, and cell pellets lysed by freeze-thaw in PBS containing 0.1% (v/v) Triton X-100. Protein samples were centrifuged to remove cell debris and the final protein concentration

on PC3 cells but not DU145 cells by flow cytometry. *D*, CD44 receptors expressed on PC3 cells are functionally competent and promote HA-induced potentiation of PC3 cell invasion. In contrast, HA was unable to potentiate DU145 cell invasion. Data shown are the mean \pm SE of three independent experiments performed in triplicate. Statistical significance between data points was determined using Student's *t* test. **, $P < 0.01$.

determined by bicinchoninic acid assay (Pierce, Rockford, IL). Proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels and then blot-transferred to nitrocellulose membranes. Membranes were blocked with 10% marvel in PBS for 1 h, incubated with primary antibody in PBS for 45 min, and washed in PBS containing 1% marvel/0.05% Tween 20 (3×15 min washes). Finally, membranes were incubated for 45 min with appropriate secondary antibodies diluted in PBS, followed by three further washes as above. Specific protein bands were detected using SuperSignal West Pico Chemiluminescence system (Pierce).

Invasion Assays. Chamber inserts (12- μ m pore size, Costar) were coated with 500 μ l of Matrigel alone (or supplemented with 100 μ g/ml of HA-sodium salt, molecular weight M_r 7.57 $\times 10^8$; protein contamination <0.01%, Calbiochem, La Jolla, CA) at a concentration of 0.222 mg/ml in phenol red-free-DMEM with 1% L-glutamine and dried overnight under sterile conditions. Cells were harvested, washed, and resuspended in phenol red-free-DMEM to give a final cell density of 1×10^6 cells/ml for PC3 and 1×10^5 cells/ml for DU145 cells, respectively. In some experiments, cells were treated with blocking antibodies for 1 h at room temperature before use in invasion assays. Five hundred microliters of the resulting cell suspension were then dispensed into the top chambers whereas DMEM conditioned media (1.5 ml) were added to the bottom chambers to create a chemotactic gradient. Invasion was measured after overnight incubation at 37°C for 16 h. Top chambers were wiped with cotton wool, and invaded cells on the underside of the membrane were fixed in 100% methanol for 10 min, air dried, stained in crystal violet for 20 min, and gently rinsed in water. When dry, the crystal violet stain on membranes was eluted using a 100% ethanol/0.2 M NaCitrate (1:1) wash for 20 min and absorbance read at 570 nm using an E_{max} Precision Microplate Reader (Molecular Devices, Wokingham, United Kingdom). The percentage of invaded cells was calculated by comparison of absorbance in test samples against absorbance determined on membrane inserts that were not wiped (total cells).

Adhesion Assays. Sub-confluent cells were trypsinized, washed in adhesion media [RPMI 1640/0.1% BSA/20 mM HEPES (pH7.4)] and complete sterile saline, and resuspended at 1×10^6 cells/ml in PBS with 10 μ M fura-4 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) for 1 h at 37°C in the dark. Cells were then pelleted, washed in serum-free medium containing 1% (v/v) of Probenecid (100 mM) and incubated for 20 min in adhesion media at 37°C in the dark to permit de-esterification of the intracellular probe. Cells (3×10^5 cells/ml) were resuspended in adhesion medium and protected from the light until experimentation. hBMECs were seeded at a density of 1×10^5 cells/ml in 96-well plates (for fluorescence work) and washed with adhesion media before assay. Cancer cells were added (300- μ l cell suspension/well) to confluent endothelial monolayers and incubated for specified times at 37°C. Nonadherent cells were removed using 2×250 μ l washes of adhesion medium. Finally, plates were read in a fluorescent plate reader (Cytofluor 4000, Applied Biosystems, Warrington, United Kingdom) at 37°C using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. For antibody blocking experiments, cells were treated for 1 h at 37°C with the antibody before treatment with the intracellular fluorescent probe. In other experiments, either prostate or breast carcinoma cells or alternatively the hBMEC monolayer were treated with hyaluronidase (16 units/ml) for 1 h. Hyaluronidase-treated cells were washed twice in adhesion media before experimentation.

Statistical Analysis of Data. Differences between data points in functional assays (invasion and adhesion assays) were assessed for statistical significance using two-tailed Student's *t* test comparisons (GraphPad Prism 3.0 software).

RESULTS

The significance of CD44 expression to cancer cell adhesion to bone marrow endothelium was studied in a panel of prostate cancer and breast cancer cell lines. The expression of this type I membrane spanning hyaladherin receptor was principally characterized on two metastatic prostate cancer cell lines, the PC3 and DU145 cells derived from metastatic lesions in the bone and dura mater, respectively, and compared against the relative expression on the transformed prostate epithelial cell line PNT1-A. As shown by Western blotting (Fig. 1A), immunocytochemistry (Fig. 1B) and flow cytometry analysis (Fig. 1C, top panel), high CD44 expression was detected in PC3 cells and was

shown to be uniformly distributed on the cell membrane. Conversely, the expression of CD44 on DU145 cells was characterized as low to negligible; Western blotting failed to detect CD44 expression on DU145 cells whereas immunocytochemistry and flow cytometry analysis did reveal weak and focal expression of CD44 on the cell surface of DU145 cells. The differential expression of CD44 on PC3 and DU145 cells was confirmed by further immunocytochemistry (Fig. 1B) or flow cytometry (Fig. 1C, bottom panel) examining the binding of fluorescently labeled HA (FITC-HA). Consistent with the differential expression of CD44, we detected a strong pericellular distribution of bound FITC-HA on the surface of PC3 cells, but no FITC-HA binding was detected on the DU145 cells. Finally, we conducted invasion assays to determine whether the differential expression of CD44 on these cell lines affected their response to extracellular HA. As expected, the presence of HA increased the invasiveness of PC3 cells 2-fold, increasing the basal rate of invasion from $6.5 \pm 1.0\%$ to $12.5 \pm 2.0\%$ ($P < 0.01$; $n = 3$). Treatment of HA with hyaluronidase before its addition to the Matrigel attenuated the invasion of the PC3 cells confirming the dependence of HA in this response (data not shown). Consistent with the absence of CD44 expression, the addition of HA to the Matrigel had no effect on the invasion of DU145 cells (Fig. 1D). Therefore, the CD44 receptor expressed on the surface of PC3 cells is functionally competent in that it binds HA and promotes increased cell invasion.

We next examined PC3 and DU145 cell adhesion to a monolayer of hBMECs. Our experiments demonstrated a time-dependent increase in PC3 cell adhesion to hBMECs, whereas DU145 cells exhibited poor adhesion to these endothelial cells (Fig. 2A). Statistically significant differences in adhesion between these cell lines were detected at

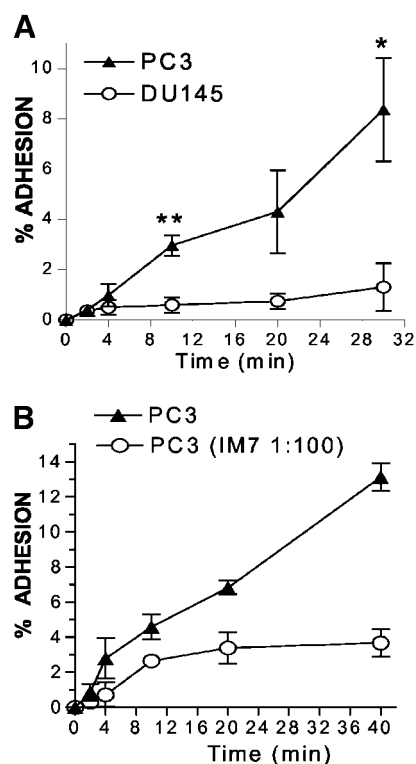


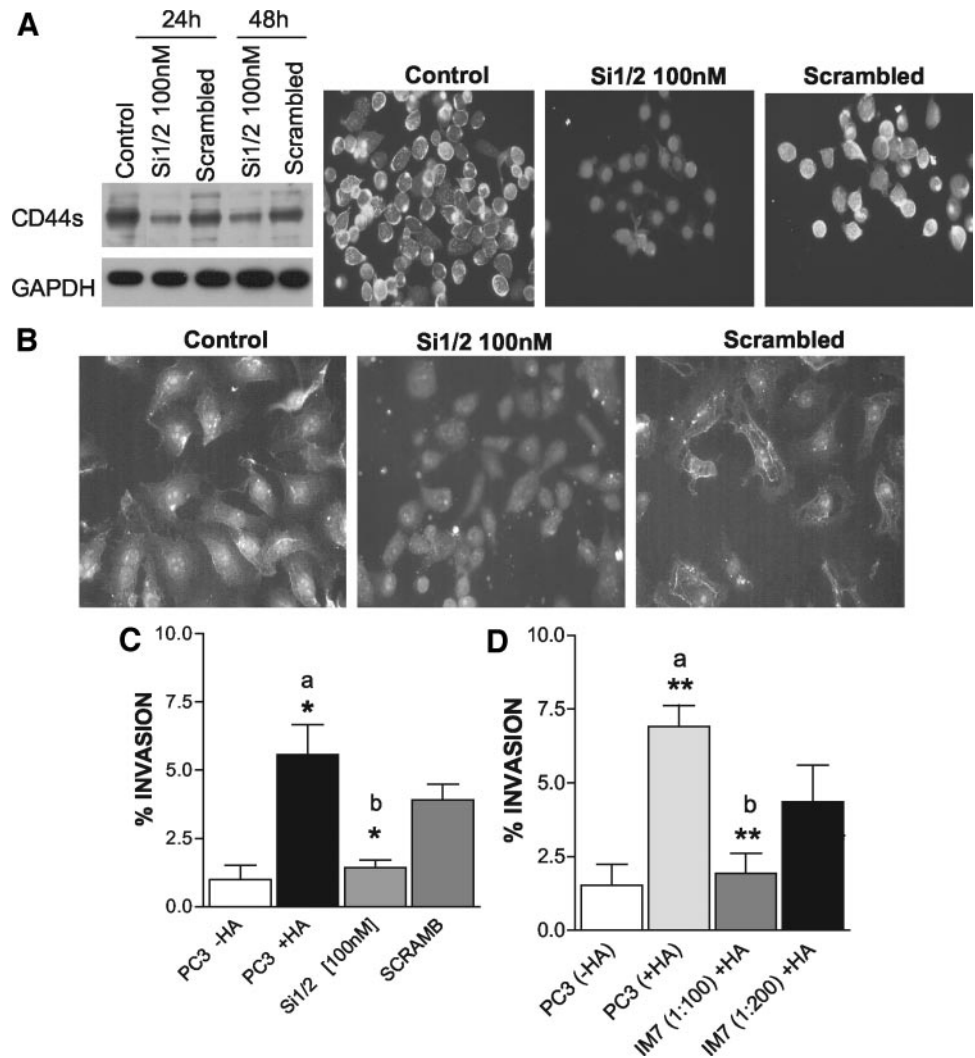
Fig. 2. CD44 expression contributes to the differential adherence of PC3 and DU145 cells to hBMECs. A, fura-4 acetoxymethyl ester-labeled PC3 cells were rapidly adherent to an immobilized and confluent monolayer of hBMECs. In contrast, DU145 cells were weakly adherent to these endothelial cells. B, the adhesion of the PC3 cells to hBMECs was inhibited by the preincubation of the PC3 cells with a 1:100 dilution of the anti-CD44 antibody (IM7 clone). Data shown for each of these experiments are the mean \pm SE of three independent experiments. Statistical significance between data points was determined using two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

10 min, reaching maximal significance at 60 min ($P < 0.001$; data point not shown). In contrast, neither PC3 cells nor DU145 cells adhered to confluent monolayers of HUVECs (data not shown). To determine whether the differential CD44 expression on these cells influenced their adhesion to bone marrow endothelium, we blocked the CD44 receptors on PC3 cells using a neutralizing antibody against human CD44 and compared their adhesion to hBMECs against that of untreated cells. Antibody-mediated blockade of CD44 receptors markedly impaired the rate of PC3 cell adhesion to bone marrow endothelium and also reduced the maximal cell adhesion attained when compared against the adhesion demonstrated for untreated PC3 cells (Fig. 2B).

RNAi Strategies to Elucidate CD44 Function in Adhesion to Bone Marrow Endothelium. To complement the pharmacological approach, we demonstrated the role of CD44 in mediating PC3 cell adhesion to hBMEC by depleting the expression of CD44 in these cells using RNAi. Oligonucleotides were designed against two nucleotide sequences within exon 3, which is conserved within the standard form of CD44 and all splice variants of this receptor. Using Western blotting, we confirmed that the expression of CD44s in PC3 cells after a single transfection (24 h) or a double transfection (48 h) with these oligonucleotides was significantly reduced (Fig. 3A). Furthermore, this RNAi strategy also decreased the expression of CD44 splice variants in PC3 cells. In contrast, treatment with a scrambled oligonucleotide at a similar concentra-

tion had no marked effect on CD44 expression. Decreases in CD44 expression were also observed using immunocytochemistry in which no CD44 expression was detectable on the cell surface of PC3 cells after combined treatment with the Si1 and Si2 oligonucleotides (Fig. 3A). However, as shown, immunocytochemistry did suggest a small decrease in CD44 expression in PC3 cells after treatment with the scrambled oligonucleotide. Selective depletion of CD44 expression using Si1 and Si2 oligonucleotides and the resulting phenotype were confirmed using two functional assays. In contrast to control cells, the capacity of PC3 cells to bind FITC-HA was absent in cells treated with the Si1/Si2 oligonucleotides, but cells transfected with the scrambled oligonucleotide retained the capacity to bind FITC-HA (Fig. 3B). More quantitative validation of the RNAi-approach was determined using Matrigel invasion assays. As shown in Fig. 3C, HA increased the invasion of PC3 cells >4-fold relative to control cells (mean/SE of $5.57 \pm 1.1\%$; $P < 0.05$; $n = 4$). Transfection with the Si1/Si2 oligonucleotides retarded the HA-promoted invasion of PC3 cells with the effect of treatment ranging from 69 to 81% diminution of the response (mean invasion $1.43 \pm 0.27\%$; $P = 0.011$, $n = 4$). In contrast, in the four experiments conducted, the nonspecific effect of the scrambled oligonucleotide was determined as 16%, 44%, 29%, and 14.5% respectively. The mean invasion of PC3 cells after scrambled treatment was $3.93 \pm 0.58\%$ and was not statistically significant from control ($P = 0.233$, $n = 4$). To further validate the

Fig. 3. Characterization and validation of RNAi-mediated depletion of CD44 expression in PC3 cells. **A**, characterization of CD44 expression in PC3 cells after mock transfection with OptiMEM, cotransfection with Si1 and Si2 oligonucleotides at final concentration of 100 nM, and transfection with scrambled oligonucleotide (100 nM). Cotransfection was shown to selectively reduce expression of CD44s and CD44 splice variants as shown in Western immunoblotting (far left panel) and immunocytochemistry (right panels). **B**, immunocytochemistry demonstrating FITC-HA binding to PC3 cells is depleted after transfection of PC3 cells with Si1/Si2 oligonucleotides but not scrambled oligonucleotide (magnification $\times 200$). **C**, functional characterization of CD44 RNAi in PC3 cells. Transfection with Si1/Si2 oligonucleotides but not scrambled oligonucleotide reduced the HA-promoted invasion of PC3 cells. Data shown are the mean \pm SE of four independent experiments performed in triplicate. **D**, pharmacological inhibition of CD44 receptors using the anti-CD44 IM7 clone antibody produces equivalent suppression of HA-promoted PC3 cell invasion. Data shown are the mean \pm SE of three independent experiments performed in triplicate. Statistical significance between data points was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.



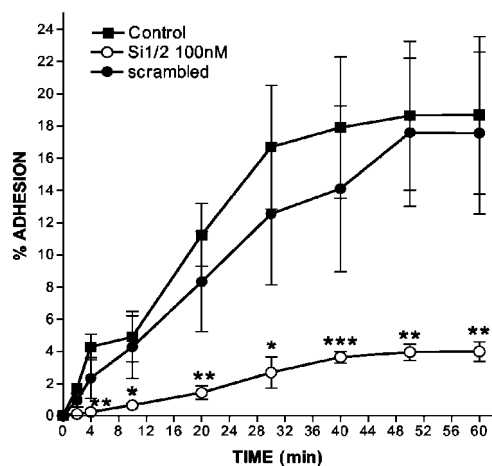


Fig. 4. Depletion of CD44 expression on PC3 cells attenuates cell adhesion to hBMECs. The time-dependent adhesion of PC3 cells is attenuated after transfection of PC3 cells with Si1/Si2 oligonucleotides at a final concentration of 100 nm but not by transfection with the scrambled oligonucleotide. Data shown are the mean \pm SE of three independent experiments. Statistical significance between data points was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RNAi approach, we compared the efficacy of depleting HA-promoted invasion by molecular inhibition of CD44 expression *versus* pharmacological inhibition of CD44 function. Preincubation of PC3 cells with increasing concentrations of the IM7 clone anti-

CD44 antibody resulted in a concentration-dependent decrease in the invasiveness of PC3 cells (Fig. 3D). The maximal effect observed using a 1:100 dilution of the IM7 antibody was comparable with that observed previously after treatment of PC3 cells with the Si1/Si2 oligonucleotides. Our CD44-targeted RNAi approach resulted in functionally/physiologically relevant CD44 depletion and our ability to obtain a large signal-to-noise ratio between the effects of CD44-directed and scrambled oligonucleotides to elucidate the function of CD44.

Role of CD44 in PC3 Cell Adhesion. RNAi was next used to validate the role of CD44 in promoting PC3 cell adhesion to hBMECs (Fig. 4). PC3 cells were either mock-transfected or transfected with either the Si1/Si2 oligonucleotides or scrambled oligonucleotide (each at a final concentration of 100 nm). Mock-transfected cells rapidly adhered to hBMECs in a time-dependent fashion, attaining a maximal adhesion of $18.7 \pm 4.9\%$ over a 60 min period ($n = 3$). Transfection with the scrambled oligonucleotide had no effect on the maximal adhesion of PC3 cells to hBMECs ($17.6 \pm 4.6\%$; $n = 3$). However, the depletion of CD44 expression attenuated PC3 cell adhesion to hBMECs. The maximal adhesion attained by Si1/Si2-treated PC3 cells was $3.98 \pm 0.59\%$ ($n = 3$). When compared against the adhesion determined for mock-transfected cells, the lower adhesion of Si1/Si2-treated cells at each time point reached statistical significance: 2 min, $P = 0.006$; 4 min, $P = 0.007$; 10 min, $P = 0.03$; 20 min, $P = 0.008$; 30 min, $P = 0.025$. These data would indicate that the expression of CD44 is necessary for optimal adhesion of PC3 cells to bone marrow endothelium.

Fig. 5. RNAi-mediated depletion of CD44 expression in breast cancer cells reduces invasiveness and attenuates breast cancer cell adhesion to hBMECs. A, characterization of standard form and splice variant CD44 expression in a panel of breast cancer cell lines. Expression of *M*_{85,000} and high molecular weight variants of CD44 was confirmed in MDA-MB-231 and MDA-MB-157 cells. B, validation of depleted CD44 expression in MDA-MB-231 cells after cotransfection with Si1 and Si2 oligonucleotides at final concentration of 150 nm using immunocytochemistry to detect cell surface CD44 expression (top panel) or FITC-HA binding (middle panel, magnification $\times 200$) or flow cytometry to detect cell surface CD44 expression (bottom panel). Transfection with the scrambled oligonucleotide (150 nm) had minimal effects on CD44 expression relative to OptiMEM-transfected controls in each of these techniques. C, immunoblotting conducted on protein lysates extracted from mock-treated, Si1/Si2-treated and scrambled oligonucleotide-treated MDA-MB-231 cells confirming Si1/Si2-mediated loss of CD44 standard and CD44 splice variant expression in these cells. D, depletion of CD44 expression on MDA-MB-231 cells after transfection with Si1/Si2 oligonucleotides (150 nm) but not scrambled oligonucleotide reduces the HA-promoted invasion of MDA-MB-231 cells. Data shown are the mean \pm SE of three independent experiments performed in triplicate. E, depletion of CD44 expression on MDA-MB-231 cells after transfection with Si1/Si2 oligonucleotides (150 nm) but not scrambled oligonucleotide reduces adhesion to hBMECs. Data shown are the mean \pm SE of three independent experiments performed in triplicate. Statistical significance between data points was determined using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

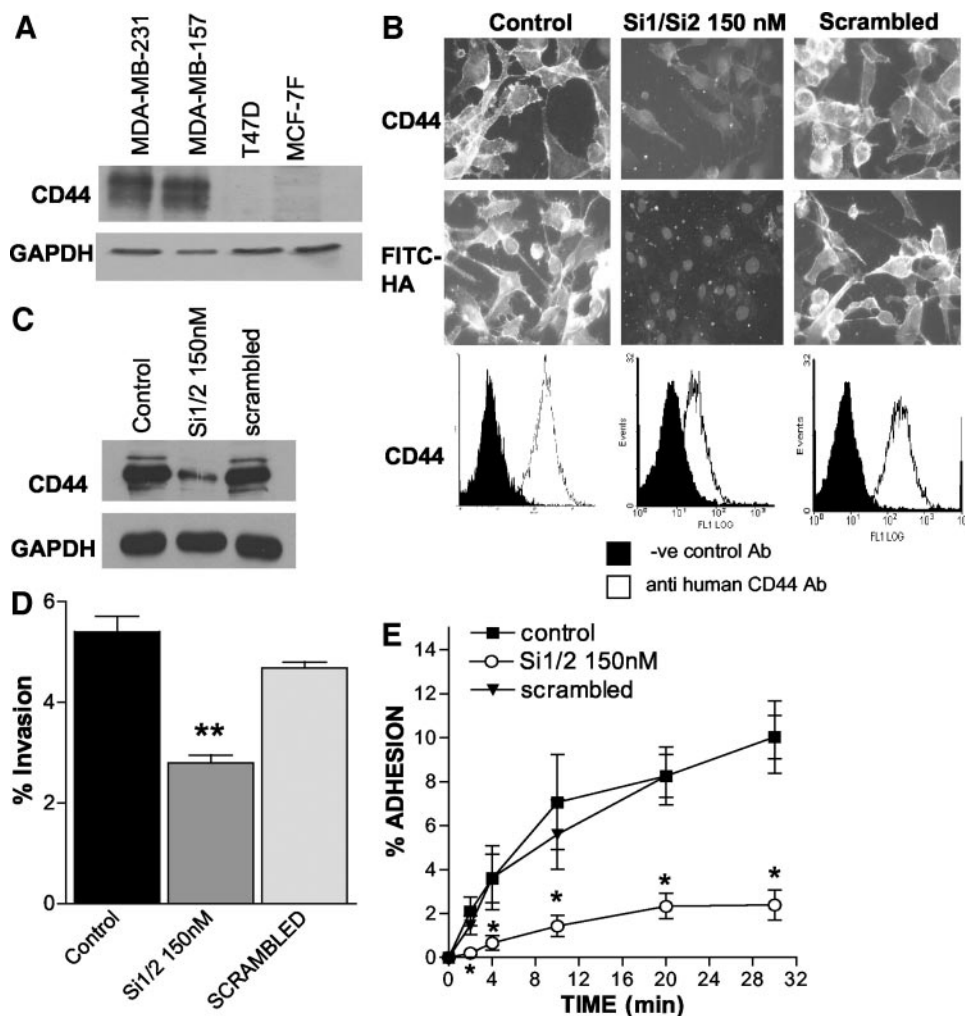


Table 1 Effect of RNAi-mediated depletion of CD44 expression on the adhesion of MB-MDA-157 cells to hBMECs

Time	MB-MDA-157		
	Optimem-transfected	Si1/Si2-transfected	Scrambled transfected
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	9.9 ± 2.96	0.92 ± 0.12 *	2.09 ± 0.65
4	16.35 ± 1.21	1.62 ± 0.72 †	8.95 ± 4.2
10	28.77 ± 4.46	4.51 ± 0.35 †	18.43 ± 1.75
20	37.48 ± 7.65	6.59 ± 0.64 †	19.97 ± 1.12
30	48.4 ± 3.23	6.1 ± 0.73 ‡	33.1 ± 4.31 *

NOTE. Data presented is the mean ± SE of three independent experiments. Statistical analysis of the data was performed using two-tailed Students *t* test.

*, *P* < 0.05.

†, *P* < 0.01.

‡, *P* < 0.001.

Role of CD44 in Breast Cancer Cell Adhesion. Bone metastasis is also prevalent in advanced breast cancer. Therefore, four breast cancer cell lines were also examined for CD44 expression to determine whether CD44 expression may also contribute to the adhesion of breast cancer cells to hBMECs. High levels of expression of CD44 standard form and splice variants were detected on the pro-invasive MB-MDA-231 and MB-MDA-157 cell lines by Western blotting (Fig. 5A). RNAi was used to reduce CD44 expression on these cells. As shown for the MB-MDA-231 cell line using immunocytochemistry, expression of CD44 and FITC-HA binding was reduced after treatment with the Si1/Si2 but not the scrambled oligonucleotides at a final concentration of 150 nM (Fig. 5B, top and middle panels). The loss of CD44 expression only on the cell surface of Si1/Si2-treated cells was also confirmed by flow cytometry (Fig. 5B, bottom panels). Similarly, immunoblotting demonstrated selective depletion of CD44 standard form and splice variant expression in MB-MDA-231 cells transfected with the Si1/Si2 oligonucleotides (Fig. 5C). In addition, the HA-promoted invasion of MB-MDA-231 cells was also abrogated after transfection with the Si1/Si2 oligonucleotides (*P* = 0.0016, *n* = 3), but transfection with the scrambled oligonucleotide was devoid of effect (*P* = 0.173, *n* = 3; Fig. 5D). Finally, adhesion assays were conducted to determine the effect of CD44 depletion on the adhesion of MB-MDA-231 cells to hBMEC monolayers (Fig. 5E). Mock-transfected MB-MDA-231 cells rapidly adhered to hBMECs in a time-dependent fashion attaining a maximal adhesion of $10.03 \pm 1.65\%$ over a 30 min period (*n* = 3). The kinetics of MB-MDA-231 cell adhesion to hBMECs after transfection with the

scrambled oligonucleotide was equivalent to mock-transfected cells. In contrast, transfection with the Si1/Si2 oligonucleotides reduced the maximal adhesion of MB-MDA-231 cells to $2.4 \pm 0.68\%$ (*n* = 3), a difference that was statistically significant from the adhesion of mock-transfected or scrambled oligonucleotide-treated cells. Similarly, the adhesion of the MB-MDA-157 cell line to hBMECs was also inhibited by depletion of CD44 expression using the RNAi approach (Table 1).

Two breast cancer cell lines, T47D and MCF-7, exhibited low to negligible expression of CD44 by Western blotting (Fig. 5A). To further demonstrate the role of CD44 in cancer cell adhesion to bone marrow endothelium, we transfected these cells to express CD44 in an attempt to promote a “gain of function” in adhesion assays. As shown in Fig. 6A, the resulting expression of CD44 in T47D cells was correlated directly with the amount of DNA used in the transfection mixture. CD44 expression in transfected T47D cells and its functional capacity to bind FITC-HA was confirmed by immunocytochemistry (data not shown). Overexpression of CD44s increased the invasion of the T47D cell line in HA-enriched Matrigel by 4.8-fold over control values (*P* = 0.002; *n* = 4; Fig. 6B). Subsequently, we demonstrated that the rate of and maximal-attained adhesion of T47D cells to hBMECs was potentiated by the increased expression of CD44s in these cells (Fig. 6C; e.g., >5-fold potentiation at the 20-min time point). In addition, there was a correlation between the magnitude of the gain in adhesion observed in these assays and the concentration of plasmid DNA used to transfect these cells (Fig. 6C).

To demonstrate that these observations were not cell line specific, similar experiments were conducted on the MCF-7 (breast) and DU145 (prostate) cell lines. Similar to our results with T47D cells, the adhesion of both the DU145 cell line (Fig. 6D) and the MCF-7 cell line (data not shown) to hBMECs was increased by >5-fold after transfection-mediated expression of CD44.

CD44-Dependent Hyaluronan Presentation by Cancer Cells Mediates Adhesion. Additional experiments were undertaken to determine how CD44 expression mediates the adhesion of prostate and breast cancer cells to hBMECs. Previously, the retention of a HA coat was implicated in PC3 cell adhesion to bone marrow endothelium (9, 10). We adopted the hypothesis that the differential expression of CD44 on PC3 and DU145 cells may impact on the ability to maintain a pericellular HA coat. Accordingly, we sought to confirm the importance of pericellular HA in mediating PC3 cell and MB-MDA-231 cell adhesion to hBMECs by repeating adhesion assays after treatment

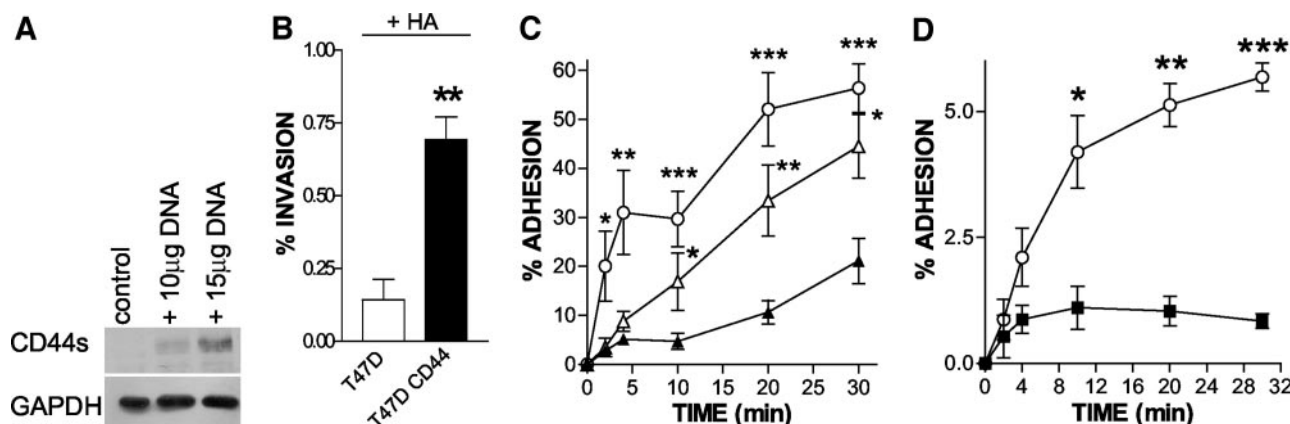


Fig. 6. Overexpression of CD44 potentiates the adhesion of breast cancer and prostate cancer cells to hBMECs. A, elevated expression of CD44s in T47D cells after transient transfection demonstrated by immunoblotting. B, overexpression of CD44s after transfection (15- μ g plasmid DNA) potentiates the invasion of T47D cells in HA-enriched matrices. Data shown are the mean \pm SE of three independent experiments, performed in triplicate. C, overexpression of CD44s in T47D cells after transfection with either 15- or 20- μ g plasmid DNA increases their adhesion to hBMECs. Data shown are the mean \pm SE of three independent experiments. \circ , T47D-CD44 (20 μ g); \triangle , T47D-CD44 (15 μ g); \blacktriangle , T47D. D, overexpression of CD44s potentiates the adhesion of DU145 prostate cancer cells to confluent monolayers of hBMECs. Data shown are the mean \pm SE of four independent experiments. Statistically significant differences between data points in functional experiments were determined by Students *t* test. \blacksquare , DU145 control; \circ , DU145-CD44 (20 μ g). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

of these cancer cells with hyaluronidase. As shown in Fig. 7A, the adhesion of PC3 cells to hBMECs was abrogated after treatment of PC3 cells with hyaluronidase at all time points studied. Similarly, the treatment of MB-MDA-231 cells with hyaluronidase also reduced the adhesion of this cell line to hBMECs (Table 2). In contrast, treatment of hBMECs with hyaluronidase had no effect on PC3 cell adhesion (Fig. 7B) or MB-MDA-231 cell adhesion (data not shown). Therefore, HA retention on the surface of cancer cells but not the receptive bone marrow endothelium would appear to underpin the adhesion of the prostate and breast cancer cells.

Finally, we determined whether CD44 expression on the endothelial cells might underpin the preferential HA-mediated adhesion of prostate and breast cancer cells to the bone marrow endothelium over other endothelial cells. CD44 expression was detected by immunoblotting in protein lysates extracted from both hBMECs and HUVECs (Fig. 8A, left panel). However, immunocytochemistry and flow cytometry only detected CD44 expression on the cell surface of hBMECs (Fig. 8A, right panels). Conversely, CD44 expression in HUVECs was only detectable by immunocytochemistry after permeabilization of the cell membrane (Fig. 8B, left panel). Similar results regarding differential distribution of CD44 in these cell lines were obtained when analyzed by flow cytometry (Fig. 8B, right panel). In addition, we demonstrated that the CD44 receptor expressed on the cell surface of hBMECs was capable of binding

Table 2 Effect of hyaluronidase treatment on the adhesion of MB-MDA-231 cells to hBMECs

Time	Control	Hyaluronidase-treated
0	0.0 ± 0.0	0.0 ± 0.0
2	2.63 ± 0.67	0.64 ± 0.03*
4	5.31 ± 1.09	1.47 ± 0.27*
10	5.43 ± 0.47	1.89 ± 0.14†
20	8.82 ± 1.09	1.85 ± 0.22†
30	8.76 ± 0.78	1.53 ± 0.54†

NOTE. Data presented is the mean ± SE of three independent experiments. Statistical analysis of the data was performed using two-tailed Student's *t* test.

*, *P* < 0.05.

†, *P* < 0.01.

FITC-HA and therefore in the active state (data not shown). To determine whether the differential CD44 expression on the cell surface of hBMECs and HUVECs may contribute to the selective adhesion of PC3 cells to bone marrow endothelium, we used RNAi to deplete CD44 expression on hBMECs and used these transfected cells to conduct adhesion assays. As shown in Fig. 8C, attenuating CD44 expression on bone marrow endothelium by RNAi resulted in a 2-fold decrease in the adhesion of PC3 cells. Similarly, the adhesion of MB-MDA-231 cells to hBMECs was also impaired by depletion of CD44 expression on the endothelium (data not shown).

DISCUSSION

Stromal HA levels are a strong, independent, and negative predictor for patient survival in breast cancer (21, 22) and prostate cancer (23). Tumor-associated HA therefore has a significant bearing on disease progression and metastasis. HA expression is absent in normal ductal epithelium but is present in breast carcinoma cells and correlates with poor differentiation, axillary lymph node positivity, and poor overall survival of the patients (21). HA levels in the urine of prostate cancer and bladder cancer patients are significantly elevated against those detected in control patients or patients with benign prostatic hyperplasia (24, 25). In addition, elevated synthesis and secretion of large molecular weight HA has been detected primarily within the tumor stroma and to a lesser degree the tumor epithelial cells in human prostate biopsy samples (26). Similarly, animal models have demonstrated that the metastatic potential of mouse mammary carcinoma cells or human breast carcinoma cells increases in association with increased HA synthesis, retention of pericellular HA matrices, and increased expression of HA-binding sites (27, 28).

Advanced prostate and breast cancers are each associated with the prevalence of metastatic deposits in bone. Access of cancer cells to the bone matrix is likely to result from selective adhesion to and extravasation across the endothelial cell layer lining the bone marrow sinusoids. HA has been implicated recently in promoting the adhesion of circulating prostate cancer cells to bone marrow endothelial cell monolayers (9). PC3 cells that exhibit high metastatic potential *in vivo* and demonstrate strong adhesion to bone marrow endothelium exhibited evidence of HA retention, elevated HA synthesis, and elevated expression of two isoforms of HA synthase, HAS2, and HAS3. In contrast, HA synthesis and HA synthase expression were considerably lower in DU145 cells and LNCaP cells, consistent with the diminished adhesion of each of these cell lines to bone marrow endothelium. Transfection of DU145 and LNCaP cells that resulted in the overexpression of HAS2 and HAS3 increased HA synthesis, promoted retention of HA on the cell surface, and increased their capacity to adhere to bone marrow endothelium (10). Similarly, inhibition of HAS2 and HAS3 activity in PC3 cells using antisense oligonucleotides diminished HA synthesis and their adhesion to BMECs.

The aim of the current study was first to identify the receptor

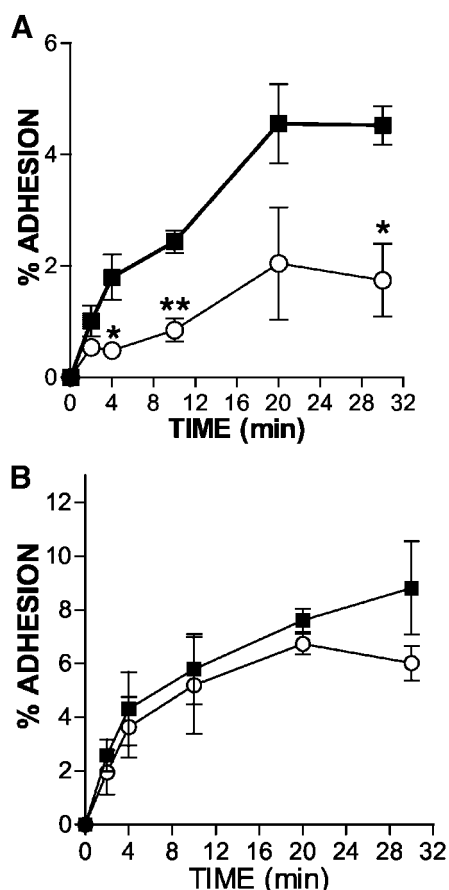


Fig. 7. Hyaluronidase treatment of PC3 cells but not hBMECs attenuates cancer cell adhesion to hBMECs. A, the time-dependent adhesion of PC3 cells to confluent hBMECs monolayers was attenuated by the preincubation of the cancer cell with hyaluronidase. ■, control; ○, + hyaluronidase (PC3 cells). B, in contrast, preincubation of hBMECs with hyaluronidase under similar experimental conditions had no effect on the adhesion of PC3 cells to these endothelial cells. Each figure shown is the mean ± SE of three independent experiments. Statistically significant differences between data points in functional experiments were determined by Student's *t* test. ■, control; ○, + hyaluronidase (BMECs). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

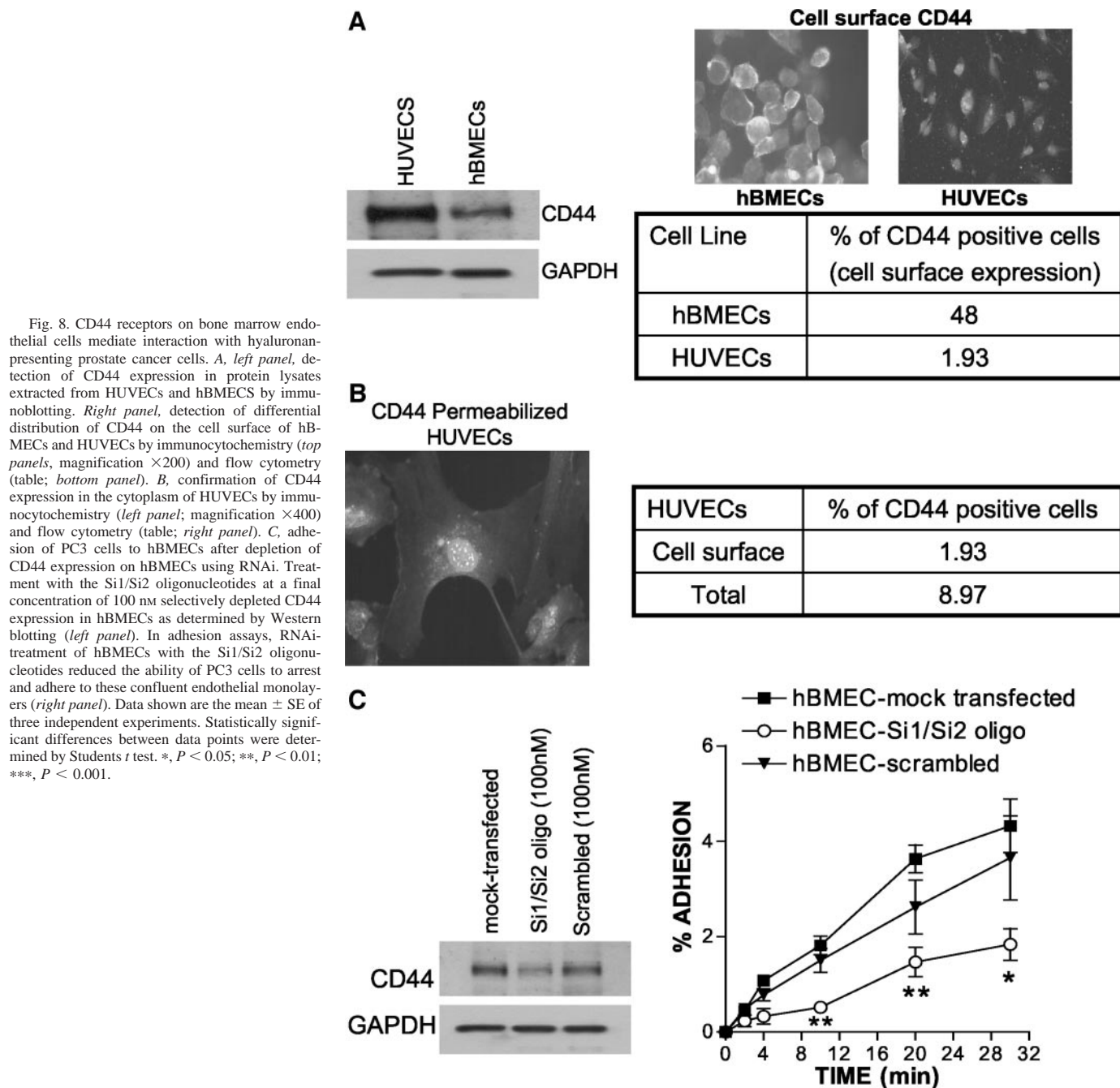


Fig. 8. CD44 receptors on bone marrow endothelial cells mediate interaction with hyaluronan-presenting prostate cancer cells. *A*, left panel, detection of CD44 expression in protein lysates extracted from HUVECs and hBMECs by immunoblotting. Right panel, detection of differential distribution of CD44 on the cell surface of hBMECs and HUVECs by immunocytochemistry (top panels, magnification $\times 200$) and flow cytometry (table; bottom panel). *B*, confirmation of CD44 expression in the cytoplasm of HUVECs by immunocytochemistry (left panel; magnification $\times 400$) and flow cytometry (table; right panel). *C*, adhesion of PC3 cells to hBMECs after depletion of CD44 expression on hBMECs using RNAi. Treatment with the Si1/Si2 oligonucleotides at a final concentration of 100 nM selectively depleted CD44 expression in hBMECs as determined by Western blotting (left panel). In adhesion assays, RNAi-treatment of hBMECs with the Si1/Si2 oligonucleotides reduced the ability of PC3 cells to arrest and adhere to these confluent endothelial monolayers (right panel). Data shown are the mean \pm SE of three independent experiments. Statistically significant differences between data points were determined by Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

underpinning the retention of HA to the cell surface of prostate cancer cells and, second, to identify the receptor facilitating the docking of the HA-presenting prostate cancer cell to the bone marrow endothelium. In addition, given the propensity of breast cancer to metastasize to the bone, we aimed to determine whether HA and HA-receptor expression would similarly influence the adhesion of breast cancer cells to bone marrow endothelium. A number of cell surface receptors for HA have been identified including CD44, RHAMM, ICAM-1, CD38, and LYVE-1; however, further intracellular receptors for HA have been proposed (cdc37, P32 and IHABP4; ref. 15). Because HA/CD44 interactions promote the arrest and adhesion of lymphocytes on endothelial cells (16, 17, 18), and more specifically, mediate the homing and transendothelial invasion of myeloma cells across the bone marrow endothelium (19), our studies concentrated on elucidating the function of CD44 in promoting the adhesion of prostate and breast cancer cells to hBMECs.

The involvement of CD44 in mediating cancer cell adhesion to bone marrow endothelium was initially investigated on two metastatic prostate cancer cell lines, PC3 and DU145. Consistent with prior reports (9, 10), PC3 cells exhibited a rapid and strong adhesion to hBMECs; however, DU145 cells were weakly adherent. Furthermore, immunoblotting, immunocytochemistry, and flow cytometry revealed that the adherent PC3 cell exhibited strong cell surface expression of the CD44 receptor whereas CD44 expression was absent from the nonadherent DU145 cells. In addition, in contrast to DU145 cells, PC3 cells were competent in binding fluorescent-labeled HA and demonstrated increased invasion in HA-enriched matrices. Furthermore inhibition of CD44 function using neutralizing antibodies or depletion of CD44 expression using RNAi attenuated the adhesion of PC3 cells to hBMECs. Accordingly, these studies provided the first evidence to implicate CD44 expression on prostate cancer cells in mediating their selective adhesion to bone marrow endothelium.

To determine whether CD44-promoted adhesion to hBMECs was unique to prostate cancer cells, we conducted additional experiments on a panel of breast cancer cells. CD44 expression was detected on two metastatic cell lines, MDA-MB-231 and MDA-MB-157 but was absent on two weakly invasive breast cancer cells, T47D and MCF-7. Consistent with our observations on PC3 cells, depletion of CD44 expression on MDA-MB-231 and MDA-MB-157 cells reduced both the invasiveness of these cells and also decreased the adhesion of these cells to hBMECs. Conversely, the adhesion of each of the CD44-negative breast cancer and prostate cancer cell lines (T47D, MCF-7, and DU145) to hBMECs was dramatically increased after transfection of these cells to express CD44. Therefore, we have been able to modulate the adhesion of prostate and breast cancer cells to bone marrow endothelium by manipulating the expression of CD44 in the cancer cells.

Treatment of either PC3 cells or MDA-MB-231 cells with hyaluronidase decreased their adhesion to hBMECs. In contrast, treatment of hBMECs with hyaluronidase had no effect on the ability of these endothelial cells to support the adhesion of either the prostate or breast cancer cell line. Our results agree with the previous findings, which proposed that the HA mediating the interaction between prostate cancer cells and the bone marrow endothelium was presented at the surface of the cancer cell and not on the receptive endothelium (9, 10, 19). This suggests that the HA retained on cancer cell surface may cross-link with HA receptors expressed on the surface of bone marrow endothelial cells. We detected high expression of CD44 on these endothelial cells and demonstrated that these CD44 receptors were able to bind fluorescent HA. In addition, parallel experiments were conducted on HUVECs to which PC3 cells demonstrate weak adherence. Although HUVECs express CD44, immunocytochemistry revealed that CD44 expression under our experimental conditions was localized to the cytoplasm and was absent on the cell surface. Therefore, the increased expression of CD44 on the surface of hBMECs may explain the preferential adhesion of PC3 cells to these cells over other vascular endothelial cells. This suggests that the adhesion of lymphocytes to endothelial cells may be dependent on the inflammation-mediated translocation of CD44 to the cell surface.

Treatment of hBMECs with hyaluronidase had no effect on the subsequent adhesion of either prostate cancer (PC3) or breast cancer (MDA-MB-231) cells, indicating that adhesion of these cancer cells is independent of a HA-enriched endothelium. However, when CD44 expression on hBMECs was depleted by RNAi, we observed a marked attenuation in the ability of PC3 cells to adhere to bone marrow endothelium. Therefore, the endothelial cell CD44 receptor would appear to function as the counter receptor that cross-links with the CD44-bound HA coat presented on the surface of the cancer cells.

Although HA/CD44 interactions effectively underpin both T-lymphocyte and cancer cell adhesion to endothelium, the current and previous studies conducted on neoplastic cells indicate that the orientation of HA presentation is different in cancer cell adhesion to that underpinning the HA/CD44-promoted lymphocyte adhesion to endothelial cells. In lymphocytic adhesion, pro-inflammatory cytokines at the site of inflammation increase the activity of HA synthase in the vascular endothelial cells to generate a HA-coated endothelium that forms the receptive milieu (29). In response to activating stimuli, the lymphocytes themselves induce the activated form of CD44 receptors on their cell surface (18, 30, 31). As a consequence, activated CD44 receptors will promote the initial tethering and capture of lymphocytes to the activated endothelium. In contrast, HA/CD44-mediated adhesion of neoplastic cells (pros-

tate/breast and myeloma) to BMECs occurs as a result of HA retention on the surface of the neoplastic cell (9, 10, 19). This study has shown that HA bound to the surface of the neoplastic cell subsequently cross-links with activated CD44 receptors expressed on the bone marrow endothelium. Therefore, it is apparent that those cancer cells that acquire elevated HAS activity and express CD44 receptors constitute an aggressive sub-population of cancer cells with increased potential to metastasize to bone.

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