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Characterization of the Expression of Variant and Standard CD44 in Prostate Cancer Cells: Identification of the Possible Molecular Mechanism of CD44/MMP9 Complex Formation on the Cell Surface

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Abstract

CD44 is a glycosylated adhesion molecule and osteopontin is one of its ligand. CD44 undergoes alternative splicing to produce variant isoforms. Our recent studies have shown an increase in the surface expression of CD44 isoforms (sCD44 and v4–v10 variant CD44) in prostate cancer cells over-expressing osteopontin (PC3/OPN). Formation of CD44/MMP9 complex on the cell surface is indispensable for MMP9 activity. In this study, we have characterized the expression of variant CD44 using RT-PCR, surface labeling with NHS–biotin, and immunoblotting. Expression of variant CD44 encompassing v4–v10 and sCD44 at mRNA and protein levels are of the same levels in PC3 and PC3/OPN cells. However, an increase in the surface expression of v6, v10, and sCD44 in PC3/OPN cells suggest that OPN may be a ligand for these isoforms. We then proceeded to determine the role of sCD44 in MMP9 activation. Based on our previous studies in osteoclasts, we hypothesized that phosphorylation of CD44 has a role on its surface expression and subsequent activation of MMP9. We have prepared TAT-fused CD44 peptides comprising unphosphorylated and constitutively phosphorylated serine residues at positions Ser323 and Ser325. Transduction of phosphopeptides at Ser323 and Ser323/325 into PC3 cells reduced the surface levels of CD44, MMP9 activity, and cell migration; but had no effect on the membrane localization of MMP9. However, MMP9 knock-down PC3 cells showed reduced CD44 at cellular and surface levels. Thus we conclude that surface expression of CD44 and activation of MMP9 on the cell surface are interdependent.

Keywords

CD44; MMP9; PROSTATE CANCER CELLS; MIGRATION; VARIANT CD44 ISOFORMS; OSTEOPONTIN

Metastasis involves the recruitment of cell adhesion molecules to the surface as well as interaction of these components with appropriate extracellular matrix that promotes cell motility (Sy et al., 1991; Nemoto et al., 2001; Spessotto et al., 2002). CD44 is a cell surface

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molecule, originally identified as a receptor for hyaluronate and later found to have affinity for other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs) (Weber et al., 1997; Thalmann et al., 1999; Yu and Stamenkovic, 1999; Cichy and Pure, 2003). Osteopontin (OPN) is an RGD-containing extracellular matrix protein, a well-characterized ligand for the integrin $\alpha v \beta 3$ and CD44 receptors in osteoclasts (Chellaiah et al., 2003a,b) and prostate cancer cells (Thalmann et al., 1999; Angelucci et al., 2002; Cooper et al., 2002; Desai et al., 2007). OPN has been implicated in cell–cell interaction, cell adhesion, migration as well as cancer growth and progression (Neame and Isacke, 1992; Thalmann et al., 1999; Nemoto et al., 2001; Desai et al., 2007). The expression level of OPN as well as de novo expression of CD44 and its variant isoforms has been associated with the metastatic potential of several tumors (Paradis et al., 1998; Rittling and Chambers, 2004; Khan et al., 2005; Rangaswami and Kundu, 2007). CD44 expression has been associated with aggressive behavior of various tumor cells. Prostate cancer cells derived from bone lesions (PC3 cells) demonstrated a rapid and strong adhesion to human bone marrow endothelial cells (hBMECs) and depletion of CD44 expression attenuated this adhesion (Draffin et al., 2004). We have previously demonstrated that OPN increases motility of prostate cancer cells through up-regulation CD44 surface expression, CD44/MMP9 interaction on the cell surface, and cell migration (Desai et al., 2007).

Migration through the extracellular matrix is mediated by MMPs, which degrade extracellular matrix components and allow cells to detach. This enables cell motility through a stromal matrix and facilitates invasion into the vascular system (Kleiner and Stetler-Stevenson, 1999). In vivo studies in melanoma demonstrated that the levels of MMP9 are significantly higher in the OPN-induced primary tumor and metastasized lung compared to control (Rangaswami and Kundu, 2007). Although PC3 cells secrete active MMP2 and MMP9 (Dong et al., 2005) in addition to synthesizing membrane bound MT1-MMP (Bair et al., 2005), MMP9 seems to be the one to regulate the migration of PC3 cells (Desai et al., 2007). Also, CD44 surface expression is considered as an important event in the activation/secretion of MMP9 and migration of PC3/OPN cells (Desai et al., 2007). After HA binding to CD44, a strong down-regulation of MMP9 mRNA and protein was detected. These findings emphasize a novel role of the HA-CD44 interaction in the context of osteoclast-like cell motility, suggesting that it may act as a stop signal for bone-resorbing cells (Spessotto et al., 2002). This also suggests a novel role for CD44 in the activation of MMP9 and cell motility. However, the mechanisms by which CD44 brings about the activation of MMP9 are unclear.

CD44 protein exists in multiple protein isoforms that are encoded by a single gene by alternate splicing of up to 10 exons, followed by extensive post-translational modifications (Choi et al., 2000; Khan et al., 2005). The conserved domain of CD44 termed standard CD44 (sCD44) is about 85–90 kDa protein product of transcription of exons 1–5 and 16–20. Exons 6–15 encode for separate CD44 isoforms labeled from CD44v1 (not expressed in human cells) to CD44v10 respectively (Cichy and Pure, 2003). Post-translational glycosylation of different CD44 variants produces proteins ranging from 80 to 200 kDa (Ponta et al., 1994). Along with standard CD44, one or multiple splice variants of CD44 may be expressed in cancer cells displaying an increased tendency for expressing larger isoforms; for example, CD44v8–10 in pancreatic carcinomas (Rall and Rustgi, 1995) and

CD44v6 in colorectal cancer (Yamane et al., 1999). The expression of CD44v6 is considered as a useful marker of tumor progression and prognosis in colorectal cancer (Yamane et al., 1999). Our aim is to identify the type of CD44 splice variants expressed in PC3 cells and address the question whether OPN had any effect on their expression. Our hypothesis was that up-regulation of one or more of CD44v and sCD44 by OPN signaling will increase MMP9 secretion and migration in PC3 cells.

We have previously demonstrated an increase in surface expression of CD44 splice variants (v3–v10) in PC3 cells over-expressing osteopontin (PC3/OPN) (Desai et al., 2007). However, the identity of the splice variants remained uncertain. Our present work aims at identifying CD44 splice variants expressed in PC3 cells and also to examine whether OPN has any role in the expression of these variants. Using specifically designed primers for CD44 splice variant sequences, RT-PCR was performed in PC3 (control cells expressing vector DNA) and PC3/OPN cells. Subsequently, the translation and surface expression of CD44 isoforms and sCD44 was analyzed in these cells. Although several ligands have been attributed to sCD44, no specific ligands have been identified for variant CD44 (Sleeman et al., 1997). In this study we showed an increase in the surface expression of variant (v6 and v10) and sCD44 in PC3/OPN cells. This suggests that OPN may be a ligand for these variant isoforms in PC3 cells.

The cytoplasmic domain of CD44 possesses serine phosphorylation sites which are responsible for CD44 activation. Of the six serine residues in human CD44, only three are conserved in humans and other mammals. Those are, Ser 316, 323, and 325. Serine 325 is the principle CD44 phosphorylation site and that mutation of this site blocks CD44-mediated migration. However, mutation does not affect the binding of CD44 to hyaluronan (Peck and Isacke, 1998). Using peptide fragments with phosphorylated serine residues, we examined whether constitutive activation of serine at these sites would have any effect on the ability of CD44 to induce migration in PC3 cells. Also we addressed whether these effects were concomitant with changes in MMP9 activity which has a critical role in the migration of PC3 cells.

MATERIALS AND METHODS

REAGENTS

Antibodies to CD44v4, CD44v5, CD44v7, CD44v8-v9, and CD44v10 were purchased from Bender Medsystems, Inc. (Burlingame, CA). sCD44 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). GAPDH and CD44v6 antibodies were purchased from Abcam, Inc (Cambridge, MA). Cy2- and Cy3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-chicken Zip-1 antibody was provided by Dr. Renty Franklin (University of Maryland, Dental School, Baltimore). MMP9 activity detection kit was purchased from Biomol International LP (Plymouth Meeting, PA). Polyacrylamide solution and protein estimation reagents were purchased from Bio-Rad. Accuprime DNA *Taq* polymerase high fidelity kit was purchased from Invitrogen Life Sciences (Carlsbad, CA). SiRNA transfection kit was bought from Ambion (Austin, TX). RNeasy Midi kit was bought from Qiagen (Valencia, CA). All other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO).

cDNA CONSTRUCTS, CELL LINES AND CULTURE

Prostate cancer epithelial cells (PC3, CRL-1435; ATCC; Manassas, VA) were transfected with OPN cDNA (PC3/OPN) and vector without insert (pCEP4) with the use of Lipofectamine 2000 (Invitrogen Life Sciences) following the manufacturer's instructions. Individual clones that expresses maximum amount of OPN were generated as described previously (Desai et al., 2007) and used for these experiments. The MMP9 SiRNA expression vector was generated using GenScript Corporation Services (Piscataway, NJ) as described previously (Desai et al., 2007). PC3 cells were transfected with the SiRNA constructs and pRNA-CMV3.1-Neo vector (as vector control) using a silencer SiRNA transfection kit. Three SiRNA constructs were generated to allow selection of one that has best possible silencing effect. Scrambled RNAi construct was used as control for SiRNA constructs. pRNA-CMV3.1-Neo vector was used for cloning. Nucleotides were synthesized and the target sequences for each SiRNA construct were as follows: (1) hMMP9_162 (length 66): GGATCCTGTAAACCATAGCGGTACAGGTTTGATATCCGACCTGTACCGCTATGGTTACACGAAGCTT; (2) hMMP9_1147:GGATCCTTGTGCGCTGTCAAA GTTCGAGTTGATATCCGCTCGAACTTTGACAGCGACAACGAAGCTT; (3) hMMP9_1677: GGATCCACTTGTGCGCGATAAGGAAGGTTGATATCCGCCTTCCTTATCGCCGACAA GTCGAAGCTT. The following scrambled RNAi sequences were cloned and cells transfected with this construct were used as negative control: GGATCCTCGCTTACCGATTGAGAATGG TTGATATCCGCCATTCTGAATCGGTAAGCGACGAAGCTT.

Individual stable PC3 clone that exhibited maximum reduction in endogenous MMP9 levels for the construct hMMP9_1147 and the stable clone that express the highest levels of OPN (denoted as PC3/OPN) (Desai et al., 2007) were generated and used for the experiments shown in Figures 1B, 2, 6, and 7. Cells transfected with vector (pCEP4) are used as control for PC3/OPN cells and is represented as PC3 (lane 1) in Figures 1B and 2. Cells transfected with SiRNA constructs to MMP9 are indicated as 162, 1147, and 1677 (Fig. 6). PC3 cells transfected with pRNA-CMV3.1-Neo vector is used as a control for cells transfected with SiRNA to MMP9 constructs (Fig. 6). PC3 cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) media containing 5% FBS at 37°C (Desai et al., 2007).

PRIMER FOR CD44 ISOFORMS

Nucleotide sequences of CD44 exons 7–15 (which code for CD44 splice variant isoforms v2–v10) were obtained from the web database of the European Bioinformatics Institute (www.ebi.ac.uk). CD44v1 primers were not made since this isoform is non-existent in human cells. The primers made encompassed about 11–15 forward 3' and reverse 5' nucleotides of the concerned exon. Sequences for the primers are provided in Table I.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AMPLIFICATION OF TRANSCRIPTS OF CD44 ISOFORMS IN PC3 AND PC3/OPN CELLS

RNA was extracted from PC3 and PC3/OPN cells using RNase midi kit (Qiagen). RNA was converted to cDNA using Superscript II RNase H Reverse Transcriptase kit (Invitrogen Life

Sciences). Using the above primers and cDNA from PC3 and PC3/OPN cells, RT-PCR was performed by utilizing Accuprime DNA *Taq* polymerase high fidelity kit (Invitrogen Life Sciences). RT-PCR reaction was carried out at an initial denaturing temperature of 94°C for 1 min, followed by a touch-down protocol consisting of 12 cycles for 25 s at 94°C, 30 s at 64°C minus 1°C per cycle, 30 s at 68°C; then 26 cycles of: 25 s at 94°C, 30 s at 52°C, 30 s at 68°C; finally 5 min at 68°C. Five microliters of reaction product from each sample was then subjected to electrophoresis on a 1.25% agarose gel. Gel images were taken under Sony's Alpha Imager 2000 system.

IMMUNOBLOTTING ANALYSIS

PC3 and PC3/OPN cells were washed three times with cold PBS and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin, and 2mM PMSF) and centrifuged at 15,000 rpm for 15 min at 4°C. Protein contents were measured using the Bio-Rad protein assay reagent. Equal amounts of lysate proteins were used for immunoblotting. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for Western analysis. Blots were blocked with 10% milk in PBS containing 0.5% Tween (PBS-T) for 2–3 h and then incubated with 1:1,000 dilutions of primary antibody of interest for 2–3h. After three washes for 10 min each with PBS-T, the blot was incubated with a 1:1,000 dilutions of peroxidase-conjugated species-specific respective secondary antibodies for 2 h at room temperature. After three washes for 10 min each with PBS-T, protein bands were visualized by chemiluminescence using the ECL kit (Pierce, Rockford, IL) (Chellaiah et al., 2003a).

CELL SURFACE BIOTINYLATION AND IMMUNOBLOTTING WITH STREPTAVIDIN-HRP

PC3 and PC3/OPN cells were labeled with NHS-biotin according to the manufacturer's guidelines (Pierce). Briefly, cells were incubated with 0.5 mg/ml biotin for 30–40 min at 4°C and washed three times with cold PBS. Biotinylated samples were subsequently lysed and subjected to immunoblotting for CD44 variants and sCD44 as described above and previously (Chellaiah et al., 2003a).

TRANSDUCTION OF TAT-FUSED PEPTIDES AND MMP9 ACTIVITY ASSAY

TAT-tagged fragments of unphosphorylated and phosphorylated peptides of CD44 were synthesized and purified by the Biopolymer and Core Science Facility (University of Maryland, Baltimore). The peptide sequences are provided in Figure 3. Transduction of TAT-fused peptides into PC3 cells was performed as described previously (Chellaiah et al., 2003b). Untransduced cells as well as cells transduced with a non-specific TAT-fused peptide (Hsv-TK; 42 kDa) were used as controls. Each transduction was performed in triplicates. Following transduction of cells with 100 nM peptide for 12–14 h, the serum-free media was collected and concentrated. MMP9 activity was determined according to the guidelines provided by the manufacturer (Biomol International, Plymouth, MA). After the reaction, optical density was measured at 450 nm to obtain relative gelatinase activity. Standard graph was generated using the protein provided in the kit. From the standard graph, the MMP9 activity in the conditioned media was calculated and plotted (Samanna et al., 2007).

IMMUNOSTAINING ANALYSIS

To determine the surface interaction of CD44 and MMP9 in cells transduced with CD44 peptides, immunostaining was performed in cells neither fixed with 1% paraformaldehyde nor permeabilized with Triton X-100. Cells were washed with PBS and immunostained with CD44 and MMP9 as described (Chellaiah et al., 2003b). To visualize the uptake of TAT-proteins, PC3 cells were fixed with 1% paraformaldehyde and permeabilized with Triton X-100. Subsequently, cells were blocked and immunostained with an antibody to HA (1:100 dilution) as described previously (Chellaiah et al., 2003 a). The immunostained cells were viewed and photographed on a Bio-Rad confocal laser-scanning microscope. Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

TRANSWELL MIGRATION ASSAY

Chemotactic migration was performed using transwell chambers with membranes of 8- μ m pore size (Costar, Cambridge, MA). The undersides of the membranes were coated with type 1 collagen as described previously (Chellaiah et al., 2003a). Approximately 1×10^4 PC3 cells in 100 μ l RPMI medium containing 5% FBS were added to the upper chamber. The bottom chamber was filled with 600 μ l of the same media. Peptides to a final concentration of 100nM were added to the upper chamber as indicated in Figure 5. Cell migration was allowed to proceed at 37°C in a standard tissue culture incubator for 12–14h. Cells that migrated to the undersides were stained with hematoxylin stain and counted in a Nikon microscope using a 10 $\times$ objective. Data are presented as the number of migrated cells/field (mean $\pm$ SEM). All assays were performed in quadruplicates. Statistical significance was calculated as mentioned below.

STATISTICAL ANALYSIS

All values were presented as mean $\pm$ SEM. A value of <0.05 was considered significant. Statistical significance was determined by analysis of variance (ANOVA) with Bonferonni corrections (Instat for IBM, Graph pad software).

RESULTS

IDENTIFICATION OF EXPRESSION OF CD44 VARIANT ISOFORMS IN PC3 CELLS BY RT-PCR ANALYSIS

RT-PCR analysis in PC3 cells.—CD44 has several splice variants containing insertions in the stem area. The most basic and common one is the standard CD44 (sCD44) (Suenaga et al., 2005). Expression of certain CD44 variant (v) isoforms in tumor cells has been correlated with metastatic and proliferative activities (Welsh et al., 1995). Immunohistochemistry studies demonstrated expression of sCD44 (68.1%), v6 (36.1%), and v9 (68.1%) to be relatively more frequent than other isoforms in prostate adenocarcinoma (Takahashi et al., 1998). In order to characterize the vCD44 isoforms expressed in PC3 cells, we performed RT-PCR analysis with the cDNA prepared from PC3 cells. CD44 splice variant primers were designed based on the individual sequences of CD44 exons obtained from the European Bioinformatics Institute. Exons 6–15 encode for the CD44 splice variants

v1–v10 respectively. Since human cells do not express v1, we did not perform RT-PCR for this variant isoform. RT-PCR revealed that some CD44 splice variants were expressed in PC3 cells (Fig. 1A); specifically v4, v5, v6, v7, and v10. The gel was photographed at higher exposure to confirm the expression of v8 isoform, which is present in a very small amount (Fig. 1A, v8). These results indicate that PC3 cells express v4–v7 and v10 isoforms in addition to sCD44 (Fig. 1B; s).

Osteopontin effect on the transcription of standard and variant CD44.—As OPN has been shown to increase the surface expression of CD44 in prostate cancer cells and osteoclasts (Chellaiah et al., 2003b; Desai et al., 2007), we compared the expression levels of splice variants in PC3 (Fig. 1B, lane 1) and PC3 cells over-expressing OPN (PC3/OPN; lane 2). RT-PCR analysis demonstrated equal levels of variant isoforms including v4–v7 and v10 as well as sCD44 at the transcriptional level (Fig. 1B). These results indicate that OPN had no effect on the transcriptional regulation of CD44.

OSTEOPONTIN INCREASES THE SURFACE EXPRESSION OF STANDARD AND VARIANT (V6 AND V10) CD44 IN PC3 CELLS

Osteopontin effect on the translation of standard and variant CD44.—To further explore whether any translational regulation occurs in response to OPN expression, we then performed immunoblotting analyses on lysates obtained from PC3 (Fig. 2A, lane 1) and PC3/OPN (lane 2) cells. CD44 splice variants undergo extensive post-translational glycosylation to produce proteins with molecular mass ranging from 60 to 200 kDa. However, antibodies to specific isoforms of CD44 purchased from Bender Medsystems, Inc., recognized a single protein band of indicated vCD44 isoform. An antibody to v6 recognizes two protein bands (Fig. 2). The approximate molecular mass of CD44 is indicated in Figure 2 and in the parentheses as follows: v4 (45 kDa), v5 (60 kDa), two bands of v6 (25 and 40–50 kDa), extremely faint band of v7 (65–70 kDa), v10 (40 kDa), and sCD44 (85–90 kDa). We did not observe any significant changes in the levels of CD44 proteins (variant or standard) in PC3 (Fig. 2A, lane 1) or PC3/OPN (lane 2) cells. Figures 1 and 2A together indicate that standard and all variant isoforms detected at significant level at the transcriptional stage underwent translation and were expressed in PC3 and PC3/OPN cells independent of OPN effect.

Osteopontin effect on the surface expression of standard and variant CD44.—Our previous analyses have revealed changes in the surface levels of variant isoforms in response to OPN expression in PC3 cells (Desai et al., 2007). The identity of CD44 splice variants was not further pursued at that time due to unavailability of specific variant antibodies. In this study, we proceeded to identify the specific variant CD44 isoforms expressed on the surface using the currently available variant antibodies. Among the splice variants translated in PC3 cells, cell surface biotinylation experiment could detect only the surface expression of v6, v7, and v10 in addition to sCD44 (Fig. 2B). A significant increase in the surface expression of v6, v10, and sCD44 was observed in PC3/OPN cells (lane 2) as compared with PC3 cells (lane 1). Surface levels of v7 seem to be unaffected by OPN. Surface expression of zinc transporter ZIP-1 (molecular mass ~35kDa) has been used as a control for loading. These data suggest that CD44 isoforms v6, v7, and v10 are expressed on

the surface of PC3 cells besides sCD44. A considerable increase in the surface expression of CD44 including, standard (s), v6, and v10 was observed in PC3/OPN cells (Fig. 2B). Although variant isoforms v7/8 and 9 was shown to increase in prostate cancer (Iczkowski et al., 2003), our observations demonstrate an increase in v6 and v10 in PC3 cells. The significance of the increase in the levels of v6 and v10 is unknown. The increase seems to be OPN-dependent and it is possible that these isoforms may have a vital role in the tumor progression. Further studies of loss-of-function of these proteins using SiRNA strategy will identify the specific role of these isoforms in PC3 cell function.

EFFECTS OF CD44 SERINE PHOSPHORYLATION ON MMP9 ACTIVITY AND MIGRATION OF PC3 CELLS

Rho kinase is one of the key players in the phosphorylation of CD44 which in turn regulates the migration of osteoclasts (Chellaiah et al., 2003b). CD44-dependent cell migration is activated by serine phosphorylation of cytoplasmic domain at position Ser325 (Peck and Isacke, 1998). Therefore, first we determined the phosphorylation levels of CD44 on serine residues in PC3 cells with a phosphoserine (p-serine) antibody (Fig. 3A). A CD44 immunoprecipitate made from PC3 cells was used for this purpose (Fig. 3A, lane 1). Immunoprecipitation with a non-immune serum (NI) is used as a control for immunoprecipitation (lane 2). Serine phosphorylation of various proteins with molecular mass approximately 180, 160, 85, 72, and 68 kDa was observed. The 85 kDa protein was confirmed as sCD44 by immunoblotting with an sCD44 antibody (bottom panel in A). It is possible that 160, 72, and 68 kDa proteins are Rho kinase, ezrin, and radixin, respectively (indicated by asterisks) because coprecipitation of these proteins with CD44 immunoprecipitates were observed in osteoclasts. These proteins were phosphorylated on serine residues (Chellaiah et al., 2003b).

Serine phosphorylation at position 325 was shown to be a critical control mechanism for CD44-dependent cell migration (Peck and Isacke, 1998). In order to study the role of serine phosphorylation on the surface interaction of CD44 and MMP9, we generated peptide sequences containing serine 323 and 325 amino acids sequences as shown in panel B (Fig. 3). Peptides were tagged with the TAT-sequence obtained from the HIV-TAT protein. The capacity of TAT-sequence to penetrate cells makes it possible for the peptide to enter the cells. Peptides thus generated were either completely unphosphorylated (P1), phosphorylated at Ser325 (P2) or phosphorylated at both Ser325 and Ser323 aa residues (P3). Non-phosphorylated (P4) and phosphorylated (P5) scrambled peptides were used as controls (Fig. 3B). PC3 cells were serum-starved and subsequently treated with the peptides of interest as described in Materials and Methods Section. From an experiment with different doses of TAT-proteins, a final concentration of 100nM TAT-fused peptides was used for transduction experiments shown in Figures 4 and 5. Immunoblotting (data not shown) and immunostaining analysis (Fig. 3C) were performed to evaluate the uptake of peptides at different time intervals with an antibody to TAT as shown previously (Chellaiah et al., 2003a). Immunostaining analysis with a TAT-antibody demonstrated that the TAT-fused CD44 peptides were able to enter cells (Fig. 3C). Immunostaining of untransduced PC3 cells were used as controls for transduction. However, transduced cells demonstrated uptake of proteins within 15 min of incubation. The uptake reaches maximum level at 60–90

min and decreases after 3 h. Proteins transduced into PC3 cells were stable for 12–14 h. Similar results were obtained in the immunoblotting analysis (data not shown). Staining was not observed in untransduced cells (data not shown). Survival of the cells was unaffected by the transduced peptides for 3–14 h (Fig. 3C). Based on the specific time-course experiment, cells were transduced with TAT-proteins for 60–45 min in serum-free medium.

ANALYSIS OF THE EFFECTS OF CD44 PEPTIDES ON THE FORMATION OF CD44/MMP9 COMPLEX ON THE CELL SURFACE, MMP9 ACTIVITY IN THE MEDIUM AND CELL MIGRATION

Analysis of distribution of CD44 and MMP9 in non-permeabilized PC3 cells.—

Since CD44/MMP9 complex is critical for the migration of osteoclasts and PC3 cells (Desai et al., 2007; Samanna et al., 2007), we sought to determine the role of the serine phosphorylation of CD44 on the interaction CD44 with MMP9, MMP9 activity, and migration. We hypothesized that serine phosphorylation of CD44 is critical for its membrane localization and interaction with MMP9 on the cell surface. We have performed immunostaining analysis with an antibody to CD44 (red) and MMP9 (green) in PC3 cells transduced with the CD44 peptides as shown in Figure 3. Surface interaction of CD44 and MMP9 was determined in transduced and untransduced PC3 cells. These cells were neither fixed with paraformaldehyde nor permeabilized with Triton X-100 prior to immunostaining (Fig. 4a,b) as described previously (Desai et al., 2007). Transduced peptides were indicated on top of each panel. Punctate colocalization (yellow) of MMP9 and CD44 was observed on the surface of cells untransduced (Fig. 4a–A) or transduced with peptides including P1, P4, P5, and Hsv-TK (B,E–G). A decrease in the colocalization or surface interaction of CD44 and MMP9 was observed in cells transduced with P2 and P3 peptides and the decrease was more with the P3-peptide containing constitutive phosphorylation on 323 and 325 (Fig. 4D). The decrease in the colocalization of CD44 and MMP9 is due to reduced levels of punctate and diffused distribution of CD44 (gray panels in C,D). However, very negligible changes in the surface levels of MMP9 was observed in these cells. Blotting of MMP9 immunoprecipitates with streptavidin–HRP demonstrated equal amounts of surface levels of MMP9 corroborated above observations in these cells (data not shown).

We then proceed to determine whether transduction of CD44 phosphopeptide (P3) has any inhibitory effect on any other cell surface protein. Human Zip-1 is a cell surface zinc transporter protein which was shown to be expressed ubiquitously on the surface of prostate cancer cells. MMP9 is a zinc-dependent endopeptidase. Therefore, we determined the surface localization of both MMP9 and Zip-1 proteins in PC3 cells by immunostaining analysis after transduction with P3 peptide which contains constitutively phosphorylated Ser323 and Ser325 residues. Untransduced cells (–) were used as control (Fig. 4b). We found diffuse and punctate distribution of MMP9 (green) in untransduced and P3-peptide transduced PC3 cells. Colocalization of MMP9 with Zip-1 (red) is very negligible in these cells. A patchy pattern of Zip-1 distribution was observed on the cell surface (bottom panels in red). Transduction of P3 peptide had no effect on the distribution of Zip-1-transporter protein or MMP9 (Fig. 4b). Transduction of CD44 peptide (P3 peptide) is not shutting down the general transport or surface expression of proteins. Therefore, the observed outcome is specific, that is, the transduced phosphopeptide has the potential to competitively block the

membrane localization of CD44. Future studies will determine the exact mechanism by which these phosphopeptides block CD44 surface expression.

Analysis of MMP9 activity in PC3 cells transduced with CD44 peptides.—We then examined the MMP9 activity in the conditioned media of cells treated with the CD44 peptides as described under Methods. Cells treated with P3 peptide had the greatest inhibition of MMP9 activity while phosphorylation at Ser325 alone had lesser effect on MMP9 activity compared with cells untransduced (–) or transduced with peptides including P1, P4, P5, and Hsv-TK (Fig. 5A).

Analysis of migration in PC3 cells transduced with CD44 peptides.—Cell migration is a function of active metalloproteinase secretion. Subsequently, migration analysis was performed to assess if changes in MMP9 activity would manipulate migration of the transduced cells (Fig. 5B). The migration profile of PC3 cells transduced with indicated peptides corresponds to the MMP9 activity under similar conditions. Our findings corroborate with those of Peck and Isacke (1998), who have shown that cell migration can be blocked by a cytoplasmic peptide containing a phosphoserine at position 325. Here we have shown that the migration and MMP9 activity depends on the surface localization of CD44. Surface localization of CD44 requires serine phosphorylation at positions 323/325.

ANALYSES IN PC3 CELLS KNOCK-DOWN OF MMP9 LEVELS

Analysis of MMP9 expression levels and activity in PC3 cells stably transfected with SiRNA and Scrambled RNAi constructs to MMP9.—We have previously shown that CD44 surface expression is an important event in the activation of MMP9 and migration of prostate cancer cells (Desai et al., 2007). CD44 null osteoclasts displayed a decrease in the levels of active MMP9 in the conditioned medium as compared with control wild-type osteoclasts (Samanna et al., 2007). In order to determine whether CD44 and MMP9 have a reciprocal functional relationship, we generated SiRNA-mediated MMP9 knock-down PC3 cells (Fig. 6). We have made three SiRNA constructs targeted to nucleotide regions 162, 1147, and 1622. SiRNA sequences are provided in the Methods section. A reduction in the total MMP9 protein level was observed in cells transfected with 1142 SiRNA construct. Among the individual clones tested with construct #1142, we chose a clone with maximum reduction at the protein (Fig. 6B, lane 5) and MMP9 activity in the conditioned medium (Fig. 6C, lane 4). A decrease of about $78\text{--}85\% \pm 8$ ($n = 4$) was observed as compared with untransfected (–) or scrambled RNAi transfected cells. The activity corresponds with the protein level shown in Figure 6B (lane 5). In culture, PC3 cells expressing reduced levels of MMP9 (1147 in Fig. 6A) were thinner and larger in size with a wavy plasma membrane (Fig. 6A, indicated by arrows in 1147) compared to other PC3 cells shown in Figure 6A.

Analysis of surface expression of CD44 and its interaction with MMP9 in different PC3 cells.—CD44 peptides (Fig. 5) and CD44 null osteoclasts (Samanna et al., 2007) demonstrated reduced MMP9 activity. In addition, the extracellular domain of CD44 binds metalloproteases, including MMP7 and MMP9, which cleave CD44s as well as variant isoform CD44v6 (Pacheco-Rodriguez et al., 2007). Inhibition of metalloprotease blocks

transcriptional activation of CD44 (Okamoto et al., 2001). Based on these observations, we proposed that PC3 cells expressing reduced levels of MMP9 would express reduced levels of CD44. Therefore, we sought to determine the levels of CD44 in PC3 cells expressing reduced levels of MMP9. We determined the surface expression levels of sCD44 by surface labeling cells with NHS-biotin (Fig. 7A) and immunostaining analysis in non-permeabilized cells (Fig. 7E,F). Immunostaining analysis was performed with an antibody to MMP9 (green) and CD44 (red) in cells neither fixed with a fixative nor permeabilized with a detergent (Fig. 7E,F). Equal amount of lysate protein (200 µg) from cells surface labeled with NHS-biotin was immunoprecipitated with an antibody to sCD44 and blotted with streptavidin-HRP (Fig. 7A). Surface expression level of sCD44 (~85 kDa) is significantly reduced in cells expressing SiRNA construct to MMP9 (Fig. 7A, lane 4; G) as compared with the untransduced (–), vector alone (V), or scrambled RNAi (Sc) transfected cells (Fig. 7A, lanes 1–3; E,F). Since there was a decrease in the surface levels of CD44, we determined the total cellular levels of CD44. Equal amount of lysate proteins (100 µg) were immunoblotted with a CD44 antibody to detect total cellular levels of sCD44. Immunoblotting analysis mirrors the surface levels of CD44 in PC3 cells expressing SiRNA to MMP9 (Fig. 7C, lane 4). A non-specific protein (~40 kDa) in the cell lysate was recognized by the CD44 antibody. Relatively equal level of this protein was observed in all the lanes (indicated by an asterisk in C). As a loading control, the blot was also stripped and reprobed with a GAPDH antibody (Fig. 7D). Statistical analysis from three experiments demonstrated a decrease of about $75\text{--}80\% \pm 6$ in the surface and $78\text{--}83\% \pm 9$ in cellular levels of CD44. Scrambled RNAi-transfected PC3 cells were used as controls. In order to corroborate that the observed decrease in the CD44 level is specific, we determined the surface levels of Zip-1 protein (B). Immunoprecipitation with a Zip-1 antibody and blotting with streptavidin HRP demonstrated equal cell surface levels of Zip-1 protein in the indicated PC3 cells (Fig. 7B). Taken together, as alluded to above, results in PC3 cells expressing reduced levels of MMP9 support our notion that MMP9 plays a role in CD44 expression.

DISCUSSION

Several variant and standard isoforms of CD44 have been observed to be up-regulated in tumors and has been considered as a possible prognostic marker (Stevens et al., 1996; Goodison and Tarin, 1998; Yamane et al., 1999). Variant isoforms of CD44 were detected by immunohistochemical and immunoblotting in prostate cancer (Iczkowski et al., 2003). Considering the importance of the association of CD44 splice variants with malignancies and the possibility of one or more of these isoforms to be involved in imparting tumorigenicity of PC3 cells, we attempted to detect the expression profile of CD44 variant isoforms of PC3 cells. We have shown here that PC3 cells exhibit transcription of exons 9, 10, 11, and 15 among the variable exons 6–15 region of the CD44 gene. These exons correspond to CD44 splice variants v4–v8 and v10. We have also detected standard CD44 (sCD44) in addition to these variant isoforms at the transcriptional level (Fig. 1). CD44v8 isoform was not analyzed further on account of its low expression levels.

The crux of the experiments is to determine the expression of profile of specific isoform(s) of CD44 at transcriptional, translational, or post-translational levels. The examination of the

effect of OPN over-expression in PC3 cells (PC3/OPN) revealed no changes in the mRNA or total cellular protein of any of the variant isoforms (v4–v7 and v10) or sCD44 (Fig. 1); whereas an increase in the surface expression of sCD44, v6 and v10 was observed in PC3/OPN cells as compared with vector transfected PC3 cells (Fig. 2). We have previously demonstrated that OPN increases surface expression of sCD44 in osteoclasts and both sCD44 and variant isoforms in human melanoma and PC3 cells (Chellaiah et al., 2003a; Samanna et al., 2006; Desai et al., 2007). Expression of certain CD44 variant isoforms in tumor cells has been correlated with metastatic and proliferative activities (Welsh et al., 1995). Osteopontin regulation of increase in the surface expression of v6 and sCD44 was observed in breast and hepatocellular cancer cells (Gao et al., 2003; Khan et al., 2005).

Characterization of individual variant isoforms with specific antibodies bought from Bender Medsystems, Inc., recognized the variant isoforms with molecular mass as indicated in the parenthesis and Figure 2: v6 (25 and 50kDa), v7 (70kDa), and v10 (30–40 kDa). One would expect a higher MW protein with variant antibodies, since the variable isoforms are further modified by extensive *N*- and *O*-glycosylation and glycosaminoglycan (GAGs) additions (Gunthert, 1993; Bourguignon et al., 1998). At present it is not known whether these CD44 are glycosylated because immunoblotting analysis with antibodies to variant isoforms specifically recognizes the isoforms exhibiting low molecular weight. Only sCD44 is identified as ~85 kDa protein. Okamoto et al. have shown expression of cleavage products with molecular mass ~25–30 kDa by Western analysis in multiple tumors excepting prostate cancer (Okamoto et al., 2001). Immunohistochemical analysis demonstrated expression of CD44 standard as well as variant isoforms 3 and 6 in prostate cancer treated by radical prostatectomy (Aaltomaa et al., 2001). Low molecular weight polypeptides of standard, v7–v9 isoforms (6–45 kDa bands) were observed at the translational level in prostate cancer by Western analysis. It was assumed that the low molecular weight isoform may be from a cleavage origin (Iczkowski et al., 2003).

CD44 cleavage can generate extracellular domain fragment as well as membrane bound C-terminal fragment (CTF; ~25 kDa) and intracellular domain (ICD; ~12 kDa) (Okamoto et al., 2001; Thorne et al., 2004). N-terminal portion generates ~37–40 kDa fragment (Nakamura et al., 2004). From our current and previous observations by others, we could recommend the following suggestions for the identification of low molecular weight isoforms of CD44: (1) The epitopes are inaccessible as a result of complex structural variability (e.g., glycosylation and GAGs addition) of the variant isoforms or recognition of the low molecular weight polypeptides of the cleavage products as suggested by others (Welsh et al., 1995; Iczkowski et al., 2003). (2) The core protein of sCD44 is 37 kDa and the apparent molecular mass of about 85–100 kDa is due to glycosylation (Nakamura et al., 2004). It is possible that variant isoforms are translated as core protein with molecular mass of about 40 kDa as observed for v6 and v10. The 25 kDa polypeptide of v6 may be the cleavage product. (3) As shown by others in prostate cancer and malignant lymphoma cells, we have also observed intact standard CD44 and low molecular weight isoforms of CD44 variants with molecular mass 40 and 25kDa (Bartolazzi et al., 1994; Iczkowski et al., 2003). These cleavage products were labeled by NHS-biotin and recognized by streptavidin–HRP in the immunoblotting analysis (Fig. 2B). Therefore, we suggest that cleavage may occur between the extracellular and transmembrane domain resulting in the formation of 25 and 40

kDa fragments of v6 and v10, respectively. The cleaved extracellular and transmembrane fragment may be accumulated in the complexes of the essential components of the extracellular matrix protein on the cell surface and labeled by NHS-biotin. These fragments hold the epitopes recognized by antibodies bought from Bender Medsystems, Inc.

Coexpression of both standard and variant isoform (v10) increased the migration capability of breast cancer cells and effectively promotes tumorigenesis in athymic nude mice (Iida and Bourguignon, 1997). The expression of distinct CD44 isoforms appears to be necessary for the progression of human tumors (reviewed in Gunthert et al., 1995). It has also been suggested that CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer cells (Friedrichs et al., 1995). The expression of CD44v6 is well known as a useful marker of tumor progression and prognosis in colorectal cancer (Yamane et al., 1999). A possible correlation between CD44 (sCD44 or CD44H) and differentiation of human prostate adenocarcinoma was observed (Takahashi et al., 1998). Soluble CD44 and variant isoform containing the v6 region were identified in cultured supernatants from all PC3 cell lines except LNCaP (Stevens et al., 1996). It is possible that an increased expression of sCD44 jointly with variant isoforms v6 and v10 (Fig. 2; Desai et al., 2007) may increase the migration capability of prostate cancer cells as observed in other cancer cells (Ponta et al., 2003; Heider et al., 2004). These may also serve as prognostic markers in prostate cancer cells. Osteopontin has been associated with malignant transformation as well as being ligand to the CD44 receptor (Thalmann et al., 1999). Although several ligands have been attributed to sCD44, no specific ligands have been identified for variant CD44 (Sleeman et al., 1997). An increase in the surface expression of variant (v6 and v10) and sCD44 in PC3/OPN cells suggests that OPN may be a ligand for these variant isoforms in PC3 cells. The exact role of variant CD44 is not yet known. As suggested, it is possible that through their binding variant isoforms may modulate the function of domains expressed on all CD44 proteins (Sleeman et al., 1997). Further studies of loss-of-function of these proteins using SiRNA strategy will identify the specific role of these isoforms in the enhancement of metastatic and invasive capability of prostate cancer cells.

Studies from our laboratory and others have demonstrated that CD44 functions as a docking molecule for MMP9 on the cell surface and suggests a mechanism for CD44-mediated cell migration (Yu and Stamenkovic, 1999; Karadag et al., 2005; Desai et al., 2007; Samanna et al., 2007). Osteopontin increases the migration of PC3 cells and osteoclasts by increasing the surface expression of sCD44 and CD44/MMP9 complex formation on the cell surface. MMP9 activity, OPN and CD44 expression are linked to prostate cancer progression and metastasis (Thalmann et al., 1999; Aaltomaa et al., 2000; Ishimaru et al., 2002; Hara et al., 2008). Peck and Isacke (1998) demonstrated that Ser325 is the principle CD44 phosphorylation site and peptides containing a phosphoserine at residue 325 are efficient blockers of CD44-mediated cell migration (Peck and Isacke, 1998). We have used similar strategy to identify the blocking role of CD44 peptides in CD44/MMP9 complex formation on the cell surface. CD44 peptides (Fig. 3B) conjugated with either penetratin or TAT sequences are translocated or transduced efficiently in cultured PC3 cells. These peptides function as efficient blockers of CD44/MMP9 complex formation on the cell surface, MMP9 activation, and migration in PC3 cells (Figs. 4 and 5). We have previously demonstrated the requirement of CD44 in the activation of MMP9 in osteoclasts isolated from CD44 null

mice. CD44 null osteoclasts demonstrated a remarkable reduction in the secretion of active-MMP9 (Samanna et al., 2007). We have corroborated our previous observation that CD44/MMP9 complex formation is critical in the activation of MMP9. Phosphorylation of CD44 plays a role in the surface expression of CD44 which is critical in the interaction of CD44 with MMP9 and activation of MMP9 (Figs. 4 and 5).

Formation of the CD44 fragment containing ICD as a result of cleavage of CD44 by metalloproteases has a role in the regulation of transcription of CD44 itself (Okamoto et al., 2001). It seems MMP9 has role in the regulation of cellular levels of CD44 by cleavage of CD44. To address the functional implication of MMP9 in the regulation of CD44 levels, we generated PC3 cells expressing reduced levels of MMP9 (Fig. 6). Our observations indeed have demonstrated that knock-down of MMP9 in PC3 cells has direct effect on the total cellular and surface levels of CD44 (Fig. 7). The biological function of CD44 differs depending upon the cleavage sites and proteinase involved. MT1-MMP also has been shown to play a role in the cleavage and shedding of CD44 (Nakamura et al., 2004). Moreover, domain swapping between MT1-MMP and other MT-MMPs revealed that the ability of PEX domain (hemopexin-like domain) to bind CD44 is conserved (Suenaga et al., 2005). It is possible that CD44 may have affinity to PEX domain of MMP9 since it functions as a docking molecule for MMP9 on the cell surface (Yu and Stamenkovic, 1999; Desai et al., 2007). Taken together, a decrease in the surface expression of CD44 in PC3 cells knock-down of MMP9 (Fig. 6) and a decrease in the activation of MMP9 in cells null for CD44 (Samanna et al., 2007) or transduced with CD44 peptides suggest a possible reciprocal relationship between these proteins. Further studies will be necessary to establish the function of MMP9 in regulation of CD44 levels. Loss-of-function of CD44 variant isoforms and MMP9 using RNAi sequences and targeting of pathways involved in CD44/MMP9 complex formation using small molecular peptide inhibitors may provide opportunities for therapeutic intervention.

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Abbreviations used:

OPN	osteopontin
Hsv-TK	human simian virus-thymidine kinase
TAT	transactivator peptide with transforming properties
MMP9	matrix metalloproteinase-9
SiRNA	small interference ribonucleic acid
MT1-MMP	membrane type 1-matrix metalloproteinase, $\alpha v\beta 3$, vitronectin receptor

HA hemagglutinin

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Fig. 1.

A: RT-PCR analysis of expression of standard (s) and variant (v) CD44 in PC3 cells. RT-PCR was performed using the primers listed in Table I. The products were analyzed on 1.5% agarose gels. The expression profile of CD44 variant isoforms (v2–v10) is shown. DNA reference ladder (100-bp) is shown in the first lane (MW). B: RT-PCR analysis in PC3 (lane 1 in each panel) and PC3/OPN (lane 2 in each panel) cells. Expression of standard (s) as well as variant (v) isoforms of CD44 including v4–v8, and v10 was observed in these cells. The results shown are representative of four independent experiments.

Fig. 2.

Determination of cellular and surface levels of standard and variant CD44. A: Immunoblotting analysis of lysates made from PC3 and PC3/OPN cells with indicated variant (v4–v7 and v10) and standard (s) CD44 antibodies. Approximate molecular mass of the protein band (kDa) recognized by respective antibody is provided below each panel. B: Surface expression analysis of standard (s) and variant (v) CD44 in PC3 and PC3/OPN cells. Cells were surface labeled with NHS–biotin. Equal amount of lysate protein (150 µg) was immunoprecipitated with indicated antibody and blotted with streptavidin HRP. ZIP-1 (30–35 kDa) was used as a loading control for surface proteins (B). PC3 and PC3/OPN cells are shown in lane 1 and lane 2 of each panel, respectively (A,B). The results shown are representative of three independent experiments.

Fig. 3.

A: Immunoblotting analysis of serine phosphorylation of CD44 in PC3 cells. Lysates (~150 µg) made from PC3 cells were immunoprecipitated with an antibody to CD44 (lane 1) or non-immune serum (NI, lane 2). Immunoblotting was performed with phosphoserine (p-serine) antibody. Serine phosphorylation of sCD44 (~85 kDa) and other proteins with molecular mass approximately 180, 160, 72, and 68 kDa was observed (indicated by asterisk). The results shown are representative of three independent experiments. B: Amino acid sequences corresponding to the phosphorylation sites (S323 and S325) of CD44 are shown. Five peptides (non-phosphorylated (NP) and phosphorylated (PP); P1-P5) were made as shown in B. Scrambled phosphopeptides were used as controls. CD44 peptides (shown in red) were tagged with the TAT-sequence of HIV-TAT protein. C: Confocal microscopy analysis of uptake of transduced proteins in PC3 cells. To determine the uptake and stability of transduced proteins, PC3 cells were immunostained with an antibody to TAT after transduction with a TAT-fused CD44 peptide (P3). Representative examples of cells transduced with TAT-fused protein at different time intervals (15min, 1 h, 3 h, and 14 h) are shown. Very negligible staining was observed in untransduced cells immunostained with a TAT antibody (data not shown).

Fig. 4.

a: Immunostaining analysis of the effect of transduction of TAT-peptides on the surface interaction of CD44 and MMP9 in PC3 cells. Confocal analysis of distribution of CD44 (red) and MMP9 (green) in cells transduced with peptides (P1–P5) and Hsv-TK is shown. The non-specific Hsv-TK peptide is used as a control for transduction. Untransduced cells are represented as (–). Immunostaining with a species-specific non-immune serum was performed in cells transduced with Hsv-TK (H). Yellow color indicates colocalization of CD44 and MMP9 on the cell surface. Distribution of CD44 is shown (gray color) in the left

side of each panel (A–H). A significant decrease in the diffuse and punctate distribution of CD44 was observed in cells treated with P2 and P3 peptides. The results represent one of three experiments performed. b: Immunostaining analysis of the effect of transduction of TAT-peptide (P3) on the surface localization of Zip-1 (red) and MMP9 (green) in PC3 cells. Colocalization of Zip-1 transporter (red) with MMP9 (green) is very minimal or not seen on the cell surface. Cell surface distribution of Zip-1 transporter is shown separately in the bottom panel (red). Transduction of P3 peptide (B) had no effect on the surface distribution of Zip-1 or MMP9. Untransduced cells are denoted as (–). Scale bar-50 μm .

Fig. 5.

The effects of various peptides on the MMP9 activity in the conditioned media (A) and migration (B) of PC3 cells. PC3 cells were transduced with peptides (P1–P5 and Hsv-TK) as indicated in A and B. Untransduced cells were indicated as (-). To determine the MMP9 activity in the conditioned media, transduction was performed in triplicates in 6-well tissue culture dishes. Equal amount of proteins in the conditioned media was used for the MMP9 activity assay as described in the Materials and Methods Section and previously (Samanna et al., 2007). Transwell migration assay was performed in quadruplicates (B) in each

experiment. In A and B, data shown are mean $\pm$ SEM of one experiment. The experiment was repeated three times. * $P < 0.05$ and ** $P < 0.001$ versus untreated (–) as well as Hsv-TK, P1, P4, and P5 peptides transduced cells.

Fig. 6.

Analyses in PC3 cells transfected with SiRNA to MMP9. We have made three different SiRNA constructs (Si) targeted to nucleotide sequences 162, 1677, and 1147 of MMP9 to generate stable PC3 cells knock-down for MMP9. Vector DNA (V), and scrambled RNAi (Sc) construct transfected as well as untransfected (-) PC3 cells were used as controls (A–C). **A:** Analysis of the morphology of indicated PC3 cells by phase contrast microscopy (400×). MMP9 knock-down cells with 1147 SiRNA construct were thinner and larger in size with wavy plasma membrane (indicated by arrows) as compared to other cells shown in A. **B:** Immunoblotting analysis with an antibody to MMP9. Equal amounts of protein lysates (75 µg) were used for immunoblotting analyses with an antibody to MMP9 (top panel). This blot was stripped and reprobed with a GAPDH antibody (bottom panel). **C:** Analysis of MMP9 activity by gelatin zymography. MMP9 activity was determined in the conditioned media collected from indicated PC3 cells by gelatin zymography. The activity of a recombinant MMP9 protein containing pro- and active band was used as an identification marker (M, lane 1). The results shown are representative of three independent experiments.

Fig. 7.

The effects of SiRNA to MMP9 on the cellular and surface levels of CD44. A,B: CD44 or Zip-1 immunoprecipitates were blotted with streptavidin-HRP to determine the surface levels of these proteins. Immunoprecipitation with a non-immune serum (NI) was used as a control for immunoprecipitation (lane 5 in A and B). Zip-1 protein was used as a control for surface expression. A decrease in the surface expression of CD44 in PC3 cells knock-down of MMP9 (A, lane 4) is specific given that no changes in the surface expression of Zip-1 was observed in these cells (B, lane 5). C,D: Cell extracts were immunoblotted with a sCD44

antibody to detect total cellular levels of sCD44 protein. Stripping and reprobing of the same blot with a GAPDH antibody reveals equal loading of proteins (D). A non-specific protein was recognized by the CD44 antibody is indicated by an asterisk in C. E,F: Confocal analysis of surface interaction of CD44 and MMP9 in PC3 cell lines. To determine the surface localization of CD44 (red) and MMP9 (green), immunostaining was performed in cells neither fixed with paraformaldehyde nor permeablized with Triton X-100. Yellow color indicates colocalization of CD44 and MMP9 on the cell surface. Distribution of CD44 is shown at the bottom of each panel (red). Colocalization is significantly reduced in PC3 cells knock-down of MMP9 (G). The results represent one of three experiments performed.

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TABLE I.

Approximately 1 µg of Total RNA From PC3 and PC3/OPN Cells Was Reverse Transcribed and Amplified Using Forward and Reverse Primers With the Sequences Provided in the Table for the Indicated Variant (v) and Standard (s) CD44

CD44 isoform	Forward primer	Reverse primer
CD44v2	TTCAACTATTATTACACAG	AGATAATAATACTCATTACCAG
CD44v3	AATGCAACTCACACACACAG	CTGTAATTGATTATTCTTACTG
CD44v4	TTCAATCATCGTTATCACAG	AAATATGCAGAACCCATTACCAG
CD44v5	TTTAACCATCATCACAGCAG	CCGACCGCCCATCTTGCTTAC
CD44v6	AACTGATATTCTTCTCACAG	CTTGTTAAACCATCCATTACCAG
CD44v7	CTCAAACTGCATGGTCACAG	AAAATCTCAGAGGGCTATTAC
CD44v8	ACTAATATTGATTCCTTCAG	CTGAGCATCGTTATTCTTAC
CD44v9	TTCATTCTCTCATTTGAAACAG	CTAGGTTTTATAATCCTTACTG
CD44v10	CTGATTCACCTCCACACAG	GATAATAAATGCCAAATTACCTG
sCD44	AAAGGAGCAGCACTTCAGGA	TGTGTCTTGGTCTCTGGTAGC