Overexpression of Vimentin Contributes to Prostate Cancer Invasion and Metastasis via Src Regulation

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Abstract. A significant proportion of prostate cancer patients treated with curative intent develop advanced disease. At a fundamental biological level, very little is known about what makes the disease aggressive and metastatic. Observational pathology reports and experimental data suggest that an epithelial-mesenchymal transition (EMT) is involved in prostate cancer invasiveness. The mechanism by which vimentin promotes prostate cancer cell invasion and metastasis was examined. The highly metastatic human prostate epithelial cell line PC-3M-1E8 (1E8-H) and the low metastatic line PC-3M-2B4 (2B4-L) were used for comparative proteomic analysis by two-dimensional gel electrophoresis, followed by matrix-assisted laser desorption/time of flight mass spectrometry (MALDI-TOF-MS). A transwell assay was performed to test cell migration and invasion and immunoblotting was used to analyze the relative expression of proteins. High vimentin expression was detected in 1E8-H compared to 2B4-L cells. Down-regulation of vimentin in 1E8-H by antisense-vimentin transfection led to a significant inhibition of invasiveness, and selective stimulation of vimentin activity in 2B4-L by delivery of recombinant vimentin promoted cell invasiveness. Vimentin activity was associated with C-src, β-catenin and E-cadherin expression. PP2, a specific inhibitor of src family kinases, reduced phospho-β-catenin expression and induce E-cadherin expression. Vimentin promotes tumor cell invasiveness and the targeting of vimentin/C-src may be a promising strategy for preventing or blocking prostate cancer metastasis.

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Prostate carcinoma is the second leading cause of cancer-related death in the United States and Europe (1), mainly due to its high potential for bone metastasis. However, the molecular mechanisms of prostate cancer metastasis are not well understood. Among the currently available techniques, cancer proteomics permits the analysis of thousands of modified or unmodified proteins simultaneously and has become increasingly popular for identifying biomarkers for the early detection, classification and prognosis of tumors, as well as for pinpointing molecular targets for improving treatment outcomes (2).

It is known that tumor progression to malignancy requires a change from an epithelial phenotype to a fibroblast or mesenchymal phenotype, a re-programming known as an epithelial-mesenchymal transition (EMT) (3-6), which is characterized by the acquisition of an invasive, mesenchymal phenotype by epithelial cells that enables their migration into a new microenvironment. E-cadherin is a prototypic type I cadherin that forms homophilic interactions through its extracellular immunoglobulin (Ig) domain, which connects to actin filaments indirectly via α-catenin and β-catenin through their cytoplasmic domains (7-9). The appropriate expression of E-cadherin on the plasma membrane is essential for cells to retain an epithelial morphology. Evidence from previous studies has shown a correlation between loss of cell–cell adhesion during EMT and decreased E-cadherin function, resulting in the aggressiveness, de-differentiation and metastasis of many carcinomas (10-17). The transgenic mouse model generated by Perl et al. has demonstrated that the loss of E-cadherin-mediated intercellular adhesion is a rate-limiting step in the progression from adenoma to invasive carcinoma in vivo (18). The intermediate filament protein (IFP) vimentin, expressed in mesenchymal cells, is a well-known marker for EMT (3). Vimentin expression and perturbation of E-cadherin-mediated cell adhesion are therefore both regarded as hallmarks of EMT-associated events. The heterogeneity and reversibility of E-cadherin protein production also remains a subject of controversy (19).
Src is a signal-transducing protein kinase that plays central roles in the control of cell growth and differentiation. Overexpression and aberrant activation of src family kinases have been identified in various human cancer cells (20, 21). It has been demonstrated that C-src kinases are enriched at the cell-cell adherent junctions of various types of cells, including hepatocytes (22). Transfection of a V-src mutant into Madin-Darby canine kidney cells led to increased association of E-cadherin with β-catenin, which caused a disruption of intercellular adhesion and an increase in vulnerability to invasion (23).

In the present study, the human prostate epithelial cancer cell line PC-3M and its clonal cells, PC-3M-1E8 (1E8-H) cells and PC-3M-2B4 (2B4-L) cells, which possess opposite metastatic potentials but similar genetic backgrounds (24), were selected as model systems for the study of the molecular events involved in prostate metastasis. Using a transfection approach the proteins associated with metastasis were screened and the potential candidates involved in regulating tumor migration and invasion were investigated. Preliminary data showed that vimentin is one of the predominant overexpressed proteins in the highly metastatic cell line 1E8-H compared to the low metastatic potential 2B4-L cells. Our studies therefore focused on the role of vimentin in mediating tumor metastasis and the possibility of cross-linking between vimentin and C-src, as well as sustained E-cadherin/β-catenin complex conformation.

Materials and Methods

Cell culture and reagents. The human prostate epithelial cancer cell lines 1E8-H and 2B4-L were obtained from the Peking University Health Science Center (Beijing, China) and were cultured in RPMI growth media supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin. The cells were kept at 37°C in a humidified atmosphere with 5% CO2. PP2 (src kinase inhibitor) was purchased from Calbiochem (La Jolla, CA, USA) and stored at –70°C.

Protein preparation for two-dimensional gel electrophoresis (2-DE). The cells were seeded onto sterile glass coverslips at 50% confluency. After 24 h, they were fixed at room temperature in 3.7% formaldehyde in PBS for 10 min and then washed three times in PBS. The cells were permeabilized for 10 min in 0.2% Triton X-100 and blocked in 1% bovine serum albumin (BSA). After a series of washes, the coverslips were incubated with mouse anti-human β-catenin (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or E-cadherin (1:100 dilution, Santa Cruz Biotechnology) for 1 h, and then washed in PBS. This was followed by a 1 h incubation with a FITC-conjugated anti-mouse IgG secondary antibody (1:100 dilution, Santa Cruz Biotechnology). Rhodamine phalloidin used to detect filamentous actin (F-actin) was obtained from Molecular Probes (Eugene, Oregon, USA). The negative control was performed without adding the primary antibody. The slides were sealed in glycerin and observed by fluorescent microscopy (Zeiss, Germany) at a wavelength of 450 nm.

Immunoblotting and co-immunoprecipitation. The cells were treated with lysis buffer containing 15% Nonidet P40 (NP40), 5 M NaCl, 2 M Tris base (pH 7.4), 0.5 M EDTA (pH 8.0), 2 mM...
phenylmethane-sulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.5 mM benzamidine, 1.5 mM sodium fluoride, 300 µM sodium vanadate and 10 mM sodium pyrophosphate. Protein concentrations were determined using a Bio-Rad protein assay. Equal amounts of protein denatured in SDS sample buffer (2% SDS, 62.5 mM Trisobase (pH 6.8), 10% glycerol, 5% β-mercaptoethanol and 0.005% bromphenol blue) were loaded onto 10% SDS-PAGE and the gels were transferred onto nitrocellulose membranes. The membranes were blocked overnight in tris buffered saline (TBS) containing 5% (w/v) powder skim milk, and after a series of washes, blots were stained with the recommended dilution of primary antibodies against vimentin (1:500), anti-C-src (Santa Cruz Biotechnology), anti-β-catenin, anti-E-cadherin, anti-phospho-C-src (Santa Cruz Biotechnology), anti-phospho-β-catenin (abcam, Cambridge, UK) or β-actin (C2; Santa Cruz Biotechnology). After further washing, the blots were incubated with a 1:1000 dilution of goat-anti-mouse IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and then developed using the enhanced chemiluminescence Western blot detection kit (Pierce Biotechnology, Inc, Rockford, IL, USA). The co-immunoprecipitation was carried out with a ProFound™ co-immunoprecipitation kit (Pierce) according to the manufacturer’s instructions.

Expression vector construction. The total RNA was isolated from the 1E8-H cells using TRIZol™ Reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The RNA (2 µg) was used for cDNA synthesis by reverse transcription. The RNA samples were incubated at 70°C for 5 min with 0.5 µg oligo deoxythymidine primers in a final volume of 10 µl and then at 37°C for 60 min in a 25 µl reaction buffer containing 125 mmol/l deoxynucleotide triphosphate, 200 U Muloney murine leukemia virus reverse transcriptase (RT) and Muloney murine leukemia virus RT buffer (Promega, Madison, USA). The obtained cDNAs were amplified by using cloning primers as follows: vimentin: 5’-CGGGATCCGGCGCCGTTGCCTTGCTCTTCTC-3’ (sense) and 5’-CGGAAAATTCCGGATCGATCCGCTGCCACTGAATG-3’ (antisense); antisense-vimentin: 5’-CTGCTCGAGCGCCCTCTTGTCGCTCTCTCC-3’ (sense) and 5’-CGGAAAATTCCGGATCGATCCGCTGCCACTGAATG-3’ (antisense). The enzyme sites for BamHI, EcoRI and Xhol are underlined. The PCR profile was 95°C for 1 minute, 94°C for 30 sec, 56°C for 1 minute and 72°C for 1 minute for 30 cycles, followed by extension for 7 minutes at 72°C. The amplified products with different restriction sites, including BamHI and EcoRI or EcoRI and Xhol, were purified using a PCR kit (New England Biolabs, Herts, UK) and ligated into the pcDNA3.1 vector (Promega) by following the instruction manual. The ligation product was transformed into E. coli DH5α competent cells. The recombinant plasmids were screened by digestion and sequenced to confirm the insert sequences of vimentin. The pcDNA3.1 plasmid with full-length vimentin was designated as pcDNAVIMs, while the pcDNA3.1 plasmid with full-length antisense-vimentin was designated as pcDNAVIMas.

DNA transfection and clone selection. To determine the effects of vimentin on invasion and metastasis in prostate cancer cells, one clone of 2B4-L cells was selected for transfection with the sense-vimentin vector and one clone of 1E8-H cells was picked up for transfection with the antisense-vimentin vector. The 2B4-L cells were transfected with 3 µg pcDNA3.1 (as a blank control) or 3 µg pcDNAVIMs, while the 1E8-H cells were transfected with 3 µg pcDNA3.1 (as a blank control) or 3 µg pcDNAVIMas according to the protocol supplied with the LipofectAMINE transfection reagent (Invitrogen Life Technologies, Inc, CA, USA). Briefly, 2x10⁵ cells were plated in 6-well dishes and incubated with the appropriate DNA and LipofectAMINE in serum-free media for 5 h, and then equal volumes of media containing 20% FBS were added. After 24 h, the media were replaced with media containing 1 mg/ml Genticin (G418). The surviving colonies were selected after 2 weeks and then maintained in 300 µg/ml G418. The positive cell clones were chosen and amplified, and neo gene expression was detected by RT-PCR using primers 5’-AGAGGGCTATTCGCTATGAGC-3’ (sense) and 5’-GCTTCAGTGCAAACTCAGAG-3’ (antisense). The blank pcDNA3.1 vector-transfected clones were established by ring cloning. Changes in vimentin levels were assayed by Western blot.

Motility and invasion assays. To evaluate the effect of vimentin on motility and invasion activity in the 1E8-H and 2B4-L cells, assays were performed in vitro by testing the ability of the cells to invade from the top well to the lower chamber. Cell motility was assessed using 24 transwell units with polycarbonate filters (Costar Corp, Cambridge, MA, USA) containing 8 µm pores. The cells were plated at 1x10⁵ cells/well in 0.5 ml serum-free media. The outer chambers were filled with 0.5 ml media containing 10% FBS. After 24 h, cells migrating to the undersurface of the filters were counted. The top surface of the membrane was gently scrubbed with a cotton swab, and cells on the undersurface were fixed in methanol and stained with haematoxylin before undergoing a series of washes. The same five microscopic fields were used to count the number of cells passing to the undersurface of each filter. For the invasion assays, the insert chambers were replaced with Matrigel (Sigma) reconstituted basement membrane layer.

Inhibition of Src. PP2, a specific inhibitor of src family kinases was used to treat the 1E8-H and 2B4-LVIMs cells. The PP2 was applied to 90% confluent 1E8-H cells at doses of 1x10⁻⁵, 1x10⁻⁴ and 1x10⁻³ M for 24, 48 and 72 hrs. Subsequently both 1E8-H and 2B4-LVIMs cells were treated with 1x10⁻⁴ M PP2 for 48 h.

Statistical analysis. All the experiments were repeated at least three times. The Student’s t-test was used to evaluate differences between experimental and control groups. Significance was defined at the level of p<0.05.

Results

Proteomic analysis of 1E8-H and 2B4-L cells. The average number of protein spots detected were 873±73 in the 1E8-H cells and 857±67 in the 2B4-L cells. The scatter plots showed good correlation between these two cell types with a correlation coefficient of 0.86, implying a similarity in the expression of most proteins, with differences in only a small number. A representative gel image was selected from the 1E8-H cells and the 2B4-L cells (Figure 1A). In total, 14 spots (including both housekeeping and differentially expressed proteins) were identified from preparative “blue silver” stained gels using MALDI-TOF-MS analysis. The
intensity of spot 2307R in the 1E8-H cells was significantly stronger compared to that in 2B4-L cells (Figure 1A) and the MALDI-TOF mass spectrum of peptides derived from 2307R is shown in Figure 1B. A Mascot search using the peptide mass fingerprint (PMF) data indicated that 19 peptides were matched with peptides from vimentin, giving sequence coverage of 38% and a summary score of 171. These results strongly suggested that vimentin was the identity of spot 2307R. To confirm the proteomic result above, Western blot and 2D Western blot analyses were used to enhance sensitivity and to effectively detect protein isoforms generated through post-translational modification. Increased expression of vimentin was detected in the 1E8-H cells compared to that in the 2B4-L cells (Figure 1C and D).

Expression of vimentin in relation to C-src, β-catenin and E-cadherin proteins. As shown in Figure 2, the blots immunoprecipitated with vimentin antibody were stained with C-src, β-catenin or E-cadherin antibodies and showed appropriate positive staining (Figure 2A). Meanwhile, the blots immunoprecipitated with C-src antibody were stained with vimentin, β-catenin or E-cadherin antibodies and showed appropriate positive staining (Figure 2B). These cross-reactions verified that vimentin was associated with C-src, β-catenin and E-cadherin.

Vimentin transfection effect on invasion and motile activity in tumor cells. The invasion of the 1E8-HVIMas cells was much lower than that of the 1E8-H cells (Figure 3A and 3C, p<0.01), whereas the invasion ability of the 2B4-LVIMs cells was noticeably higher than that of the 2B4-L cells (Figure 3A and 3C, p<0.01). Figure 3B shows the motility of the tumor cells which was represented as the mean numbers of cells counted on the undersurface of each filter per observed field (Figure 3D). The results of the motility assay were consistent with the invasion assays in the same cell lines (2B4-L vs. 2B4-LVIMs and 1E8-H vs. 1E8-HVIMas, respectively).

The effects of vimentin transfection on the expression of proteins involved in invasion. Western blot assays showed significantly decreased vimentin expression in the antisense-vimentin-transfected 1E8-H cells, designated as 1E8-HVIMas, compared to the non-transfected or pcDNA3.1 vector-
transfected cells. Meanwhile, an induced vimentin level was detected in the sense-vimentin-transfected 2B4-L cells, named 2B4-LVIMs, which was much higher than in the non-transfected or blank vector-transfected cells (Figure 4A).

The expression levels of the relevant proteins, including C-src, phospho-C-src, β-catenin, phospho-β-catenin and E-cadherin were tested, in both the 1E8-H and 2B4-L cells and in the 1E8-HVIMas and 2B4-LVIMs cells. As shown in Figure 4B, the 1E8-HVIMas cells displayed decreased expression of C-src, phospho-C-src and phospho-β-catenin compared to non-transfected 1E8-H cells, while E-cadherin protein levels increased and the expression of β-catenin protein showed no change. Induced expression of C-src, phospho-C-src and phospho-β-catenin and a reduced E-cadherin level were detected in the transfected 2B4-LVIMs cells, compared with the non-transfected 2B4-L cells. The β-catenin level remained stable after vimentin transfection (Figure 4B). Immunofluorescence assays showed that strong E-cadherin and β-catenin staining existed at points of cell-cell contact in the 1E8-HVIMas and 2B4-L cells, forming classic zipper-like structures (Figure 5A and B, arrows), whereas in the 1E8-H and 2B4-LVIMs cells, E-cadherin and β-catenin were either not visible or presented a weak staining at boundaries between opposing cells, indicating that the 1E8-H and 2B4-LVIMs cells were easy to detach.

Inhibition of C-src and the expression level of corresponding proteins involved in invasion. As shown in Figure 6A, B and C, PP2 caused dose-dependent and time-dependent inhibition of the expression of C-src in the 1E8-H cells. The lowest effective dose of PP2, 1x10⁻⁴ M, and an appropriate time point, 48 h, were used to test its effect on β-catenin and E-cadherin expression in the 1E8-H and 2B4-LVIMs cells. As shown in Figure 6D, a reduced expression of phospho-β-catenin and a remarkably increased E-cadherin expression were detected in the PP2-treated cells compared to the non-treated cells. The β-catenin level remained stable after PP2 administration (Figure 6D).
Discussion

Significant expression of vimentin was detected in 1E8-H cells (high metastatic potential) compared with 2B4-L cells (low metastatic potential). Western blot and 2D Western blot analyses further verified this result in the present study. Some investigators have also demonstrated that vimentin is highly expressed in invasive cancer cells, as determined by proteomic analysis or microarrays (28-31). Previous studies have demonstrated that vimentin is expressed in epithelial cells that undergo tumor invasion (32, 33). Lang et al. have also demonstrated that vimentin is expressed in motile prostate cell lines and positively correlates with poorly differentiated carcinomas and bone metastases (34). The important contribution of vimentin to the invasive phenotype of prostate cancer cells was demonstrated by the present transfection results. The overexpression of vimentin increased invasiveness in the stable, vimentin-transfected 2B4-LVIMs cells, while in contrast, the decreased vimentin expression in antisense-vimentin-transfected 1E8-HVIMas cells led to a significant decrease in invasiveness, as determined by Matrigel invasion assays. This was consistent with results from overexpression of vimentin in human breast cancer cells resulting in phenotypic interconversion and invasive behavior conversion (33).

The immunoblot and immunofluorescence analysis in the present study demonstrated that loss of E-cadherin activation correlated inversely with increasing vimentin expression in the stable, vimentin-transfected 2B4-LVIMs cells. Therefore, both vimentin activity and E-cadherin-mediated cell adhesion...
appear to be hallmarks of EMT-associated events implicated in cell migration and invasion. Furthermore, the data also demonstrated that vimentin and C-src kinase were physically linked and functionally coupled in the human prostate cancer cells, indicating the possibility that vimentin affected the E-cadherin/β-catenin complex via C-src.

Early work suggested that the E-cadherin/β-catenin complex was further stabilized by serine phosphorylation of E-cadherin at residues 684, 686 and 692. Structural analysis revealed that these residues only interact with β-catenin when phosphorylated (35-37). Phosphorylation of β-catenin tyrosine residue 654 by C-src causes an approximately six-fold reduction in the affinity of β-catenin for E-cadherin (38). In our study, phospho-β-catenin expression increased in the 2B4-LVIMs cells and decreased in the 1E8-HVIMas cells, while the expression of β-catenin protein in the transfected cells was the same as in the non-transfected cells. Furthermore, src inhibition reduced phospho-β-catenin expression and induced E-cadherin expression in highly metastatic cells, and could probably inhibit detachment of these cells.

There is some evidence that phosphorylation of two of the critical tyrosines of β-catenin that disrupt the core E-cadherin complex, 142 and 654, has the potential to regulate the exchange of β-catenin from its association with E-cadherin to its role in nuclear localization and transcription (39). The phosphorylation of tyrosine 654 increased the ability of β-catenin to bind the basal transcription factor TATA-binding protein and enhanced transcriptional activity of β-catenin. Thus, β-catenin levels remained stable following vimentin transfection or PP2 administration in the prostate cancer cells.

In conclusion, vimentin can promote tumor cell invasiveness by regulating the E-cadherin/β-catenin complex via C-src in prostate cancer cells. Therefore, vimentin might be a target for therapeutic agents aiming to block the metastasis of prostate cancer, either through antisense-vimentin transfection or through the use of specific inhibitors, such as C-src inhibitors.

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References