The Predominant CD44 Splice Variant in Prostate Cancer Binds Fibronectin, and Calcitonin Stimulates its Expression

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Abstract. *Background: Prostate cancer (PC) consistently overexpresses variant the (v) isoform of the cell adhesion protein CD44, and loses expression of the standard (s) isoform. Materials and Methods: We re-expressed CD44 full-length (exons 1-20) or standard (exons 1-5 + 16-20) or enforced stable RNAi against CD44v, and the examined functional effects on PC. The effect of stable knockout of calcitonin, a paracrine factor, or its receptor, on CD44 was assessed. Results: Re-expression of full-length CD44 or CD44s increased the total CD44 mRNA and CD44s protein while suppressing CD44v. These approaches, and RNAi to CD44v, decreased invasion. In adhesion assays, benign prostate cells bound mainly to hyaluronan, whereas PC lost affinity for hyaluronan but bound more strongly to fibronectin. Re-expressing CD44s restored predominant hyaluronan binding. Knockout of the calcitonin receptor in PC-3 derived cells caused marked loss of CD44v expression and reversion to CD44s expression. Conclusion: Calcitonin influenced PC's balance between CD44s and CD44v. CD44v controlled invasiveness, altered ligand binding, and provides a target for therapeutic intervention.*

The transition from quiescent to aggressive prostate cancer (PC) requires tumor cell detachment, interaction with proteins that digest the stromal matrix, migration through the matrix for local expansion, and lymphovascular intravasation for metastasis. Crucial to these processes are cell adhesion proteins such as CD44. CD44 is a family of transmembrane glycoproteins that enable homotypic cell, cell-matrix and cell-cytoskeletal interaction. CD44 has roles in embryogenesis and tumor invasion and metastasis. The CD44 gene, which maps to chromosome 11, contains 20 exons spanning 60 kB. CD44 protein is subdivided into five structural domains and can assume several isoforms. CD44 *standard* (s) mRNA comprises exons 2-5 in the extracellular N-terminal domain, plus exons 16-20, including the transmembrane region (exon 18) and tail (exons 19-20) (Figure 1A). The N-terminus is the hyaluronan receptor but also binds collagen type IV, fibronectin, laminin and osteopontin. Clustering of CD44 molecules is required to bind hyaluronan (1, 2). CD44s is expressed as a 90 kDa protein on all cells, and facilitates lymphocyte homing on cells of hematopoietic origin (1, 3). Depending on the cell's microenrivonment, splicing can include all standard exons plus intervening exons 6-15, to encode longer, *variant* (CD44v1-10) isoforms whose gene products lengthen the extracellular stem. CD44 variant expression is restricted to normal and neoplastic epithelium in a tissue-specific fashion. Overexpression of novel, single or contiguous variant exons has been described in many carcinomas (3-9).

Miyake *et al.,* in 1998, reported that transfection of a transient CD44s expression vector into the prostate cancer cell line PC-3 reduced growth *in vitro* and tumorigenicity in subcutaneous implants (3), suggesting that it suppresses metastasis during prostate carcinogenesis. CD44s can clearly function differently in other cancers, however, and promote metastasis (4, 7). We (10), and subsequently others (11-14), observed that PC loses immuno-histochemical expression of CD44s and some CD44v isoforms, particularly CD44v6. Later, using RT-PCR followed by sequencing, as well as immunostaining and Western blot analysis, we showed that primary and metastatic PC overexpressed the CD44v7-v10 sequence (Figure 1B) at both mRNA and protein levels (15, 16). Cloning of this purified PCR product into TOPO vector and sequencing disclosed total correspondence to the
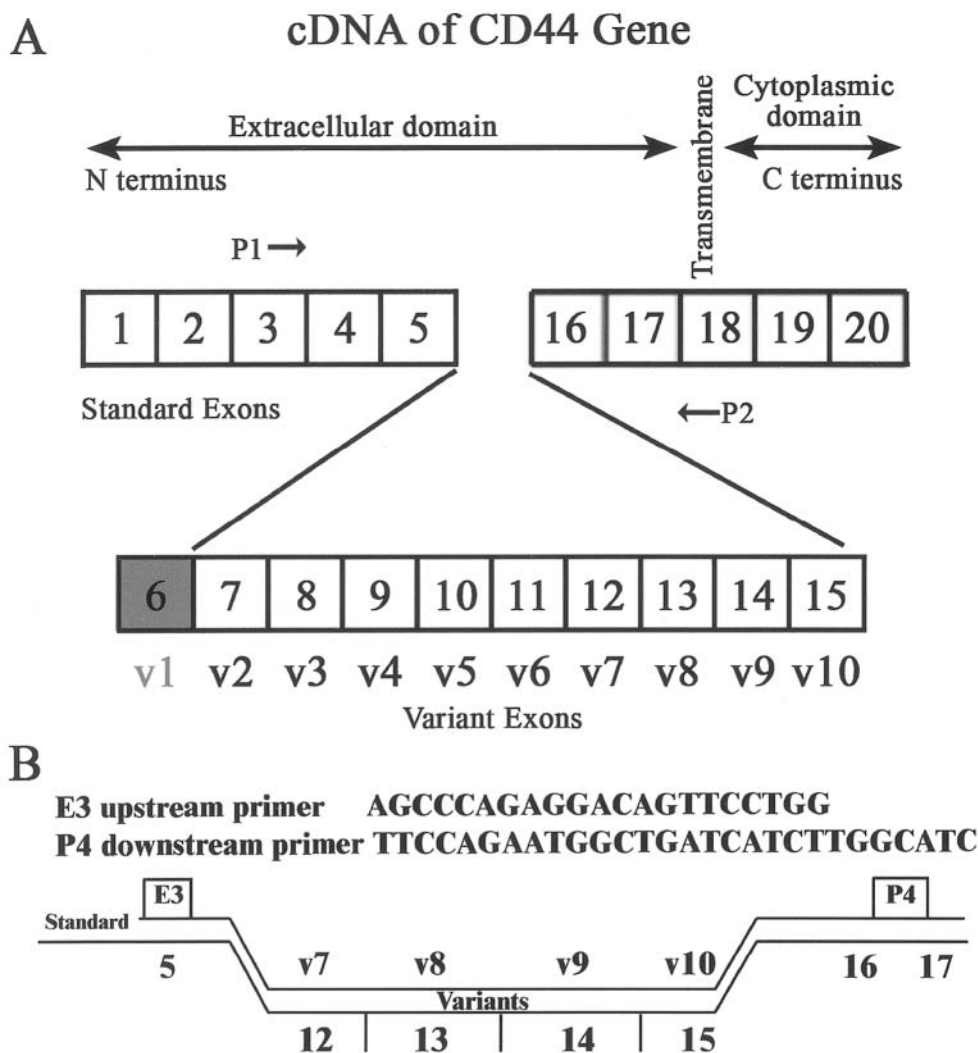
A   cDNA of CD44 Gene

Figure 1. *A. The standard form of CD44 protein comprises exons 1-5 and 16-20. The extracellular stem may be lengthened if splicing causes inclusion of up to 10 variant (v) exons. (CD44v1 is expressed in rats and mice but contains a stop codon and thus it is silent in humans.) The mRNA expression of any variant exons may be determined by PCR-amplification with primers such as P1 and P2. Cancers feature characteristic, tissue-specific overexpressed variant isoforms. In primary and metastatic prostate cancer, we discovered that the predominant CD44 form that is expressed is CD44v7-10, as part of an amplifiable 608-bp band (15, 16). B. Boxes indicate our primers E3 and P4 (9), slanted lines indicate splice sites, and the double line indicates amplified transcript.*

published CD44 v7-10 transcript (8), including the variant exons 12-15. We hypothesized that CD44v7-10 over-expression in PC played a tissue-specific, proinvasive role, comparable to the overexpression of CD44v6 in pancreatic cancer (6) and in breast cancer with increasing grade (7). We showed that partial abrogation of CD44v7-10 expression significantly reduced invasion of tumor cells into the basement membrane-like matrix, confirming that its overexpression in PC was not an epiphenomenon (16).

We here postulate that enforced expression of CD44s in the CD44s-deficient PC-3 cell line can reduce CD44v expression and inhibit PC invasiveness *in vitro*. Secondly,

using benign and cancer prostate cell lines and re-expression transfectants, the predominant stromal ligands for CD44v were determined, helping to understand the mechanism of CD44v action, with possible therapeutic implications for PC. The most aggressive cell line $G_s\alpha$-QL (17, 18), which expresses CD44v more strongly than PC-3, was additionally studied in the adhesion assays. Thirdly, some of our prior findings about calcitonin (19), a 32-amino acid peptide that is secreted in increased amounts by malignant prostate epithelium, were extended. The influence of knockout of calcitonin or its receptor on CD44s and CD44v expression was examined.
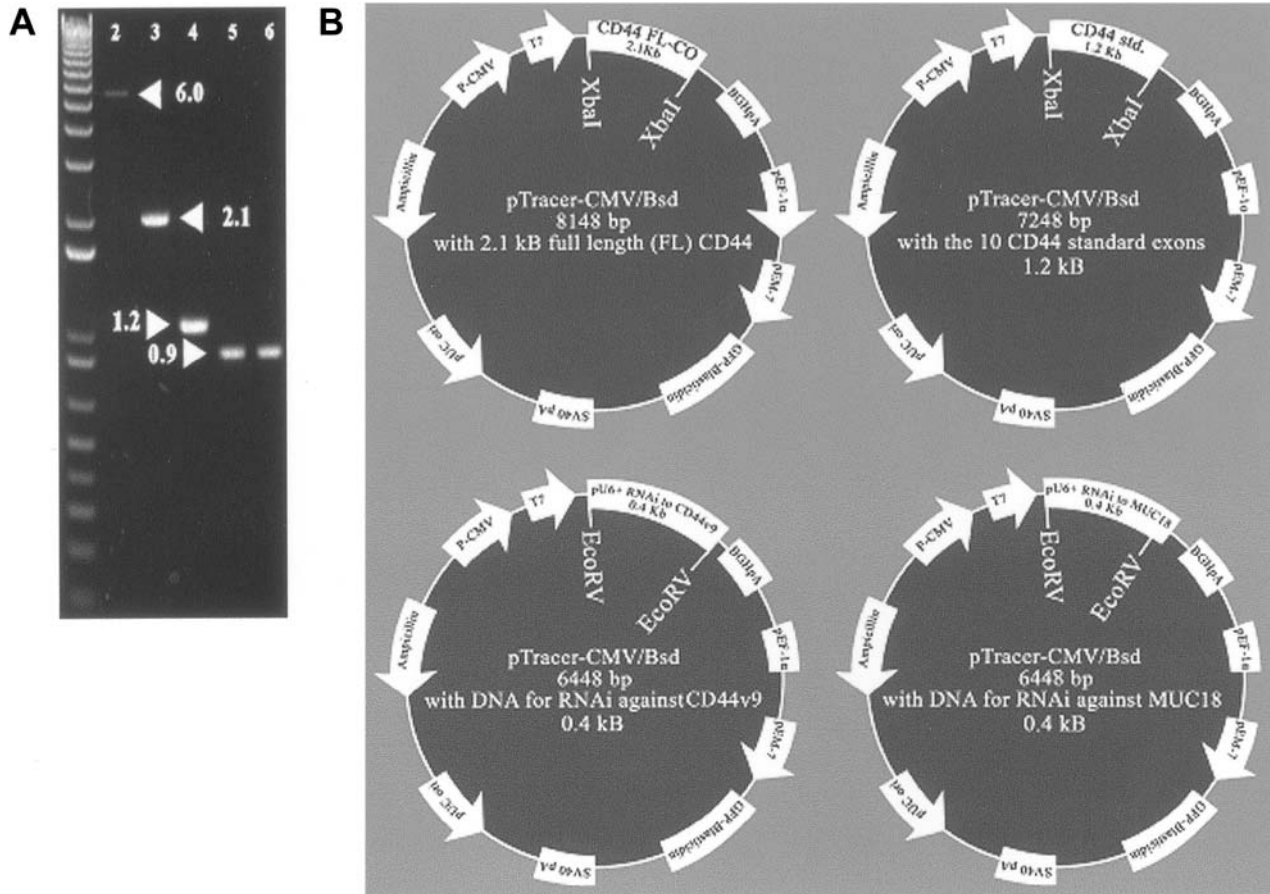
Figure 2. *DNA fragments containing CD44 used in preparing transfection constructs. A. Lane 1, kB Plus marker; Lane 2, 6.0 kB XbaI-digested and gel-purified pTracer*; Lane 3, full-length CD44, 2.1 kB, for overexpression; Lane 4, CD44 standard is 1.2 kB; Lanes 5,6, CD44v7-10, plus C-terminal standard sequence, 0.9 kB, amplified from malignant prostate from two patients. B. Our four transfection constructs were made from pTracer (Invitrogen) which has genes for GFP for visualization of transfectants and for blasticidin resistance. For overexpression, the sequence of either complete full-length CD44, or of CD44 standard, was cloned at the XbaI among multiple cloning sites under the control of the CMV promoter (PCMV) and the U6 promoter brought in from splicing the sequences out of pTOPO. For stable RNAi enforcement, the same transfection construct also was made to express CD44v9 RNAi and MUC18 RNAi constructs cloned at the EcoRV site. All constructs were confirmed by sequencing to have our overexpression or RNAi constructs in the correct orientation. Cloned plasmids were used to transfect PC-3 or $G_s\alpha$-QL cells followed by blasticidin selection. C. Pure GFP+ populations were achievable by clonal selection of transfected cells.*

Materials and Methods

Cell culture. The less aggressive LnCaP cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated in RPMI 1640 with L-glutamine, 10% fetal calf serum, and 50 U/mL penicillin, 50 μg/mL streptomycin and 20 μg/mL, fungizone at 37°C in a 5% CO_2 incubator. The invasion assays and growth *in vivo* used PC-3 cells (ATCC) that were incubated in Ham's F12-K medium plus the above supplementary ingredients. $G_s\alpha$-QL cancer cells are a PC-3 derivative stably transfected (17, 18) to express mutant, constitutively active $G_s\alpha$ protein. These cells were maintained in complete medium (RPMI 1640 with L-glutamine, 5% fetal calf serum, 12% horse serum, and the above antibiotics). For cell adhesion assays, the above three PC lines were compared. Also used were benign BPH-1 cells (Dr. Simon Hayward, Vanderbilt Univ., Nashville, TN, USA), passaged in the same medium as LnCaP.

RNAi transfectants for interference against CD44 variant. The CD44v9 interference DNA sequence was excised, together with the U6 promoter from pU6BS used previously (16), and inserted into pTracer (Invitrogen, Carlsbad, CA, USA) (Figure 2A), where its expression is driven by two promoters, CMV and U6, to produce more complete RNAi (Figure 2B). The pTracer vector was chosen because it has a strong CMV promoter, GFP to indicate successful transfection, and a blasticidin resistance gene to allow selection for positive cells. Ten μL Metafectene (Biontex, Munich, Germany) and 10 μg DNA were each diluted separately in 100 μL of sterile serum-free RPMI before mixing (20). The mixture was allowed to stand for 30 min to allow formation of Metafectene-DNA complexes (20) and the $G_s\alpha$-QL cells were transfected as previously (16). The RNAi sequence for MUC18, a cell adhesion molecule implicated in PC (21), was also excised from pU6BS (16) and inserted into the pTracer. Untreated and transfected cells were

Table I. *Matrix ligands tested in cell adhesion assays.*

Ligand	Coating concentration	Source	Vendor	Location
Laminin	20 μg/mL	human	Sigma	St. Louis, MO, USA
Collagen type IV	20 μg/mL	mouse	Becton Dickinson	Bedford, MA, USA
Fibronectin	20 μg/mL	human	Becton Dickinson	Bedford, MA, USA
Hyaluronate	2 mg/mL	rooster comb	Sigma	St. Louis, MO, USA
Osteopontin	10 μg/mL	osteopontin-GST fusion protein	Upstate Biotechnology	Lake Placid, NY, USA

analyzed by RT-PCR, using primers spanning both standard and variant regions (Figure 1A) (16), and by Western blot analysis as described below.

Re-expression transfectants, CD44 full-length and CD44 standard. CD44 DNA full-length and standard fragments are shown as cloned (Figure 2B). Full-length CD44 (clone IMAGE, 3638681/ GenBank Accession #BC004372) of 2,387 bp length was purchased from Open Biosystems (Huntsville, AL, USA). The clone has an open reading frame of 2.1 kB. The 3' end of this open reading frame sequence aligns 100% with our sequenced CD44v7-v10 from prostate cancer (16). Two PCR primers were designed for CD44 full-length amplification. At the 5' end, the Xba*ICD44 forward* contains the Kozak translation initiation sequence with an ATG start codon for proper initiation of translation (22). To this primer we have genetically engineered an *XbaI* restriction site before the Kozak translation initiation sequence. At the 3' end, we also genetically engineered an *XbaI* restriction site into the *XbaICD44 reverse* after the stop codon TAA. We have successfully used these primers in PCR reactions to amplify full-length coding region of CD44 using the Open Biosystems clone as a template. The PCR products were fractionated by electrophoresis, excised and eluted from the gel, then cloned into TOPO (Invitrogen). TOPO clones that appeared to have the CD44 full-length coding region were subjected to restriction analysis using *XbaI*. Several TOPO clones, selected on the basis of restriction analysis, were sequenced. The TOPO clone confirmed as having the correct sequence was digested with *XbaI*, fractionated by electrophoresis, grown in Stbl2 *E. coli* cells (Invitrogen), and ligated into pTracer in the correct orientation.

In prostatectomy tissues, both PC and benign prostate expressed a 1.2 kB, CD44s band. Sequencing of the 1.2 kB band in PC, however, showed that it was missing part of exon 18, the transmembrane domain. Its function in PC was uncertain, it was amplified by PCR from first-strand cDNA made from extracted total RNA. The primers used were the same as those used above for full-length CD44. The PCR products were fractionated by electrophoresis, and the 1.2 kB DNA fragment was excised and eluted from the gel. Using the *XbaI* restriction enzyme, cloning into TOPO, selection, and ligation of the 1.2 kB CD44s into the pTracer, were carried out as for full-length CD44. The correct orientation of the fragment was confirmed by sequencing. Both full-length CD44 and the CD44s clones were used to transfect PC-3 cells as described for RNAi. Clonal selection of transfected cells yielded pure GFP+ populations.

Total CD44 RNA in cell lines and transformants was quantified using equal amounts of RNA with primer set E3/P4 that spans the variant region, amplifying all CD44s and CD44v (9). The RT product was also run with primers for ribosome 18S (Applied Biosystems, Foster City, CA, USA) as a loading control.

SDS-polyacrylamide gel electrophoresis and Western blot analysis for CD44v9 or CD44s. A pellet from centrifuged cultured cells was placed into RIPA lysis buffer (Upstate Biologicals, Lake Placid, NY, USA) supplemented with fresh protease inhibitors, 2 μg/mL Pepstatin A, 1.5 μg/mL Leupeptin and 1 mM PMSF. Cell debris was removed by centrifugation. The protein lysate was treated with an equal volume of 2% SDS and the protein concentration was estimated by the Lowry method. Twenty-five μg of sample/lane was electrophoresed (15). One part of 5x sample buffer (Bio-Rad, Hercules, CA, USA) was added to four parts of the solubilized proteins. The proteins were then denatured for 5 min in a 100°C boiling water bath and 40 to 50 μL were loaded into wells of a 4% stacking gel. Five μL of Rainbow protein marker RPN 756 (Amersham Pharmacia, Piscataway, NJ, USA) were also loaded on each gel. SDS-PAGE was performed by the Laemmli method using 12% polyacrylamide gels. After electrophoresis, the protein was blotted onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad). The membrane was hybridized overnight to the primary antibody. The antibody was either the supernatant of HB-258 mouse hybridoma cells (ATCC), which produce a monoclonal antibody for CD44 variant 9, used neat, or anti-CD44s (Ventana, Tucson, AZ, USA) used at 1,1000 dilution. Goat anti-mouse IgG labeled with horseradish peroxidase (Pierce, Rockford, IL, USA) was used as a secondary antibody at 1,10,000 dilution. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce).

Cancer cell invasiveness. As previously (16), studies were performed in six-well two-tier invasion chambers (Collaborative Biomedical Products, Bedford, MA, USA). 2.5x10^5 PC-3, $G_s\alpha$-QL, or transformant cells per well were seeded in the upper insert in a serum-free basal medium (RPMI 1640 medium containing 0.1% BSA, 150 mg/mL of G418, 4 mM L-glutamine, 100 μg/mL penicillin G and 100 μg/mL streptomycin). The lower chamber contained 2 mL of 70% complete medium with chemoattractants (10% fetal bovine serum and 20% conditioned medium obtained from subconfluent cultures).

Incubations were carried out for 36 h. After this period, uninvaded cells were removed from the upper Matrigel surface using cotton swabs. The invasive cells should be situated on the outside bottom of the upper insert. The gel inserts were fixed, stained using May-Grünwald staining (EM Science, Gibbstown, NJ, USA), and mounted on glass slides. The total number of cells on the entire gel was counted. Three experimental trials were done
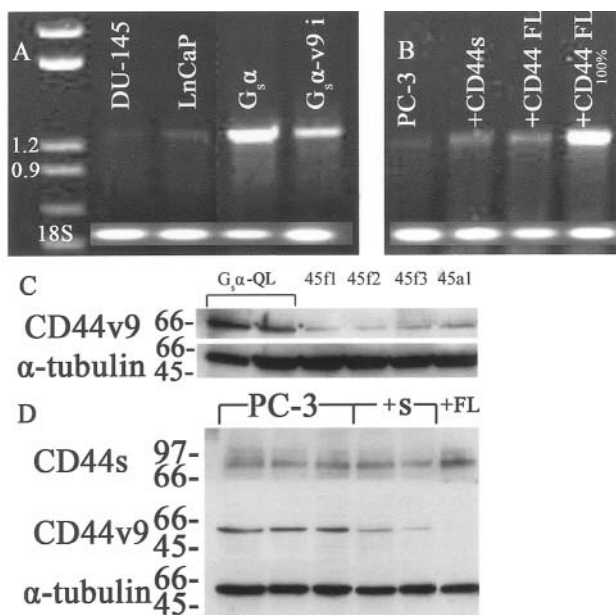
Figure 3. *A. RT-PCR products of cell lines and transfectants. The primer set E3/P4, that amplifies all standard plus variant isoforms (9), was used. Total CD44 was minimal in the DU-145 and LnCaP cell lines (lanes 2-3). CD44 RNA was greater in the more aggressive PC-3 cells and highest in the most aggressive $G_s\alpha$-QL cells. 18S RNA loading controls are shown at bottom. B. The expression of either CD44s or full-length (FL) CD44 increased the total amount of CD44 message detected, particularly when 100% transfection efficiency was achieved (lane 4). 18S RNA loading controls are shown at bottom. C. Compared with untreated $G_s\alpha$-QL cells, those of clone 45 with stable RNAi against CD44v9 had about 90% inhibition of the CD44v protein product. f1-f3, floating and a1, attached passaged cells. CD44s protein was absent in all $G_s\alpha$-QL cells (data not shown). D. Overexpressed DNA, assessed by green fluorescent protein (GFP), was present in 50-100% of the cells. Western blot analysis of PC-3 cells for CD44s showed that overexpression of the shorter CD44s (excluding part of exon 18), or CD44FL in correct orientation increased the detected CD44s. Analysis for CD44v showed that overexpression of CD44FL or CD44s greatly reduced the CD44v cleavage product detected by anti-CD44v9.*

and absolute numbers of invasive treated cells were normalized to percents of the control cells. The data are expressed as mean±SD. The significance of differences among the means of groups was tested by two-tailed Student's *t*-test.

Cellular adhesion assays. Cellular adhesion assays were carried out as described (23), using trypsinized confluent untreated BPH-1, LnCaP, PC-3 or $G_s\alpha$-QL cells, PC-3 cells overexpressing CD44s, and $G_s\alpha$-QL cells with RNAi against CD44v9. Each test condition was set in eight wells and each experiment repeated twice.

Ninety-six-well black-edged, clear flat bottomed Costar plates (Cole-Parmer, Vernon Hills, IL, USA) were coated with optimal concentrations of ligands (23), using eight wells to test each one, at 37˚C overnight as in Table I. As controls, eight wells were coated with 1 mg/mL BSA to measure baseline non-specific binding. 1×10^6 cells suspended in 1 mL PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo, Japan) for 15 min at 37˚C
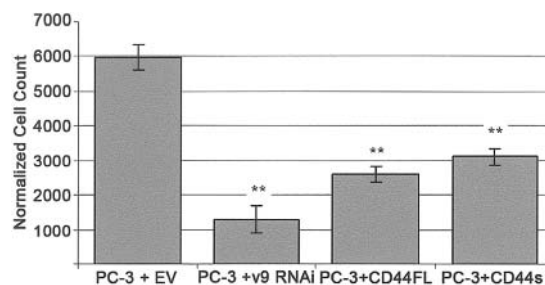

Figure 4. *Matrigel invasion assays (mean of six trials ±SD) in PC-3 cells. RNAi of CD44v9 (v9i) in 100% of cells produced a 78% decrease in invasion compared to empty-vector (EV)-transfected cells. CD44FL overexpression caused a 57% decrease in invasiveness, consistent with the concept that CD44s counteracts the production of CD44v. Overexpression of CD44 the standard (CD44s) in 100% of transfectants also produced a 47% decrease in invasiveness (**, p<0.01).*

(23). After two washes of the cells with PBS, they were added to plates at a density of 5×10^4 per well and incubated at 37˚C for 90 min. Fluorescence intensities at 530 nm were measured using a Bio-Tek FL-600 fluorescent plate reader. Non-adherent cells were removed with three PBS washes. The fluorescence intensities with PBS in the wells were measured. Adhesion was calculated (23) as, % cells bound=(100) fluorescence intensity after washing / fluorescence intensity of total cells plated.

To examine the dependency of adhesion to hyaluronan and other ligands on CD44s or CD44v, cells were preincubated with antibody that binds to the hyaluronan-binding region (clone BU75, Ancell, Bayport, MN, USA), at a density of 5 μg/10^6 cells in 1 mL PBS for 30 min at 4˚C (23). Preincubation with isotype-matched mouse monoclonal IgG (Pharmingen, San Diego, CA, USA) was used as a negative control in all trials. The preincubated cells were washed three times with PBS before being applied to the adhesion assay. To dissect out the role of integrin *versus* CD44 in fibrillar fibronectin adhesion, the cells were seeded into wells in the presence of 0.5 mg/mL of and RGD peptide, that blocks β_1-integrin binding to fibronectin, namely GRGDSP (SynPep, San Jose, CA, USA) (19). GRADSP peptide was used as a negative control. The data are expressed as mean±SD. The significance of differences among group means was tested by the two-tailed Student's *t*-test.

Calcitonin and calcitonin receptor transfectants. Previously (19), we showed that exogenous calcitonin stimulation or stimulation of calcitonin's receptor, the $G_s\alpha$ protein, or its downstream effectors increased CD44v expression and decreased CD44s. The same effect was observed with the CT+ cell line, stably transfected to produce elevated endogenous calcitonin. Taking this a step further, we recently studied CD44 expression in cells with anti-CT hammerhead ribozymes that prevent making CT (CT-) or anti-calcitonin receptor ribozymes (CTR-).

Results

CD44 silencing and overexpression. The CD44 expression was assessed in the transfectant cell lines receiving either siRNA or constitutive expression vector by analyzing the abundance
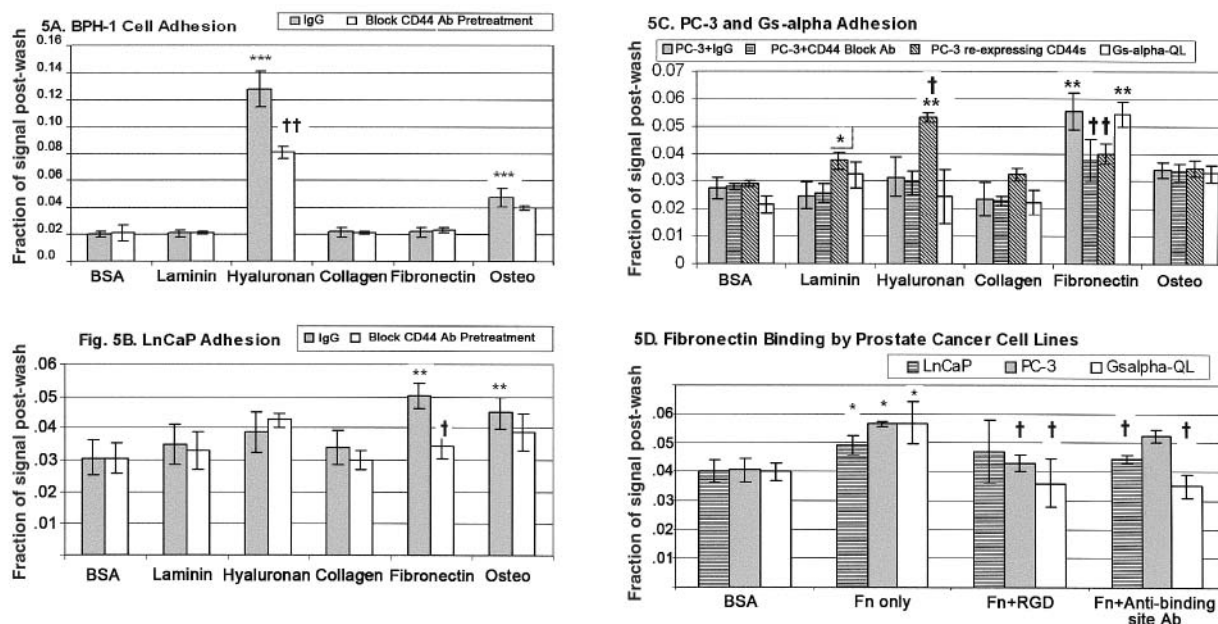
Figure 5. *The cell adhesion assays show the predominant stromal ligands bound by CD44 in benign and cancerous prostate cell lines of increasing aggressiveness. BSA-coated wells represent baseline signal. Error bars for each condition show standard deviation based on 8 wells. All experiments were repeated at least twice. Binding above baseline, ***, p<0.005; **, p<0.01; *, p<0.05. Reduction of maximal binding by antibody or RGD peptide, ††, p<0.005; †, p<0.02. A. In benign BPH-1 prostate, CD44s expression was high. Hyaluronan binding was 6x above (p<0.005), and osteopontin binding 2x above (p<0.01) that of other ligands. This hyaluronan binding is reduced by antibody (0.5 μg/10^6 cells) that blocks CD44's hyaluronan binding site (p<0.005). B. In LnCaP, the slowest growing cancer line, the binding was significantly above baseline for fibronectin (p<0.01) and, to a lesser extent, osteopontin (p<0.01). The fibronectin binding was reduced by blocking the CD44 binding site (p<0.02). C. The more aggressive PC-3 and most aggressive $G_s\alpha$-QL cells lacked hyaluronan binding, consistent with loss of CD44s expression. Fibronectin binding was elevated above baseline in empty-vector (EV) PC-3 and $G_s\alpha$-QL (p<0.005). In PC-3 cells, antibody blockage of CD44's binding site reduced fibronectin binding (p<0.005), implicating a role for the predominant CD44v. CD44s overexpression elevated binding above baseline for hyaluronan (p<0.005) and laminin (p<0.02). Compared to PC-3 EV, the hyaluronan binding was increased and fibronectin binding was reduced (p<0.005). D. Significant fibronectin binding in LnCaP, PC-3 and $G_s\alpha$-QL cells (p<0.05) was reduced by antibody blockage of the CD44v binding site or abrogated by the RGD peptide GRGDSP, blocking the β_1 integrin that is the definitive fibronectin receptor (p<0.005). This suggests CD44 functions as an accessory to $\alpha_5\beta_1$ integrin.*

of CD44 transcripts. CD44 mRNA was amplified using PCR with a primer set that spans the variant region and thus detects the total amount of CD44s and CD44v present (Figure 3A). The specificity of changes in the transcript levels was confirmed using 18S as a loading control. Initial studies examined baseline expression in DU-145, LnCaP, PC-3 and $G_s\alpha$-QL cells. The latter displayed the highest CD44 levels; however, after transfection with RNAi against CD44v9, $G_s\alpha$-QL showed markedly less CD44 RNA, suggesting that CD44v comprised most of the total RNA.

PC-3 cells expressed minimal CD44 transcript by RT-PCR (Figure 3B). PC-3 was transfected with either full-length (FL) CD44 or CD44 standard plasmids. The effects in these sublines are depicted in lanes 2-4. The PC-3 sublines receiving either CD44s or CD44FL displayed increased concentrations of CD44 transcripts. To determine the effects of these manipulations of CD44 at the protein level, CD44s and CD44v immunoreactivities were analyzed in subline lysates using Western blotting. In clone 45 of $G_s\alpha$-QL cells –

floating or attached – with stable RNAi against CD44v9, about 90% inhibition of the CD44v9 protein product was achieved, compared to the untreated cells (Figure 3C). This inhibition of CD44v was unaccompanied by changes in CD44s. In the PC-3 cells (Figure 3D), despite the low level of CD44 transcripts (Figure 2B), some protein was detected, and almost all of it was CD44v. Overexpression of CD44FL or CD44s resulted in increased CD44s at 90 kDa. Interestingly, forced expression of either CD44s or CD44FL was accompanied by suppression of the CD44v7-10 cleavage product, detected at 66 kDa.

Since our prior studies showed that silencing of CD44v expression decreased PC invasiveness (16), this effect was compared with that of forced expression of CD44FL or CD44s by Matrigel invasion assays (Figure 4). PC-3 cells overexpressing either transcript were less than half as invasive compared to empty-vector transfected PC-3 cells. Thus, CD44s apparently counteracted the role of CD44v in PC-3 invasion.
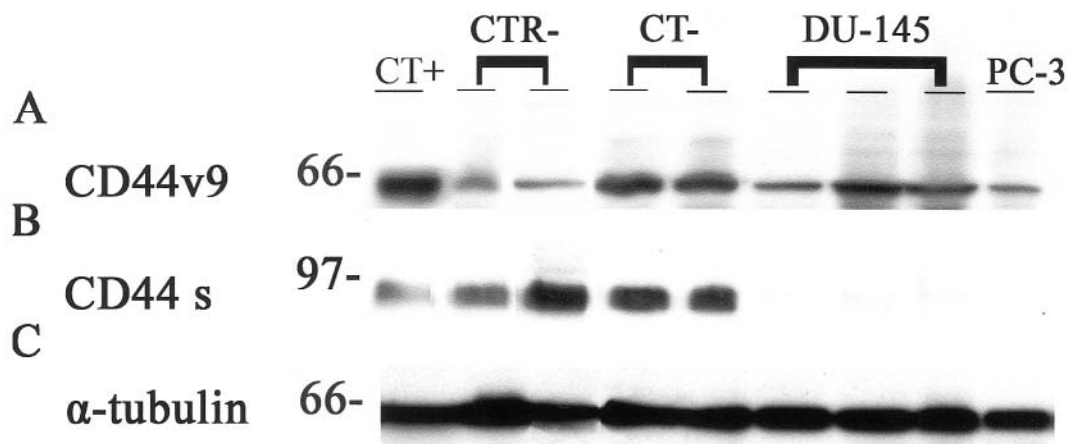
Figure 6. *Simultaneous Western blot analyses for (A) CD44v9 and (B) CD44s expressions using equal amounts of protein from the cell lines and transfectants. The constitutive calcitonin expressor CT+ showed highest detection of cleaved CD44v and minimal CD44s. In calcitonin receptor knockouts CTR–, CD44s predominated. Calcitonin knockouts CT– showed an even balance between CD44v and CD44s. Two androgen-independent cell lines, DU-145 and PC-3, expressed moderate CD44v and almost no CD44s. PC-3 is the parental cell line of the metastasis-derived cells used to make the three transformants. The findings provide further evidence that the calcitonin system influences CD44 mRNA splicing toward CD44v. (C) Reactivity for α-tubulin as a loading control.*

Cellular adhesion assays. Cell adhesion assays demonstrated that benign BPH-1 cells bind six times more strongly to the definitive CD44 ligand hyaluronan than to the other ligands tested, a likely consequence of their high CD44s expression (Figure 5A). This binding can be partly inhibited by pretreatment with blocking antibody to the CD44 binding site. The BPH-1 cells also bound significantly to osteopontin (Figure 5A). In the cancer cell lines, the hyaluronan-binding affinity was insignificant. The least aggressive cells, LnCaP, in which minimal CD44s expression was retained, showed significant affinity for fibronectin and osteopontin (Figure 5B). The use of blocking antibody showed the fibronectin interaction to be CD44-dependent. In the PC-3 and their very aggressive derivative $G_s\alpha$-QL cells (Figure 5C), binding to fibronectin was two to four times greater than to the other ligands tested. In PC-3 cells with re-expression of CD44s, however, fibronectin binding was reduced while hyaluronan binding was increased (Figure 5C). To distinguish whether fibronectin binding was attributable to CD44 *versus* integrins, CD44 binding site blocking antibodies or RGD peptide, respectively, were used (Figure 5D). Fibronectin binding by LnCaP and PC-3 cells showed significant dependence on the CD44 binding site; complete abrogation of binding was achievable by RGD peptide blocking.

CD44 in calcitonin-negative or calcitonin receptor-negative transfectants. The CT– and CTR– transfectants were compared with the CT+ transfectant, which we had previously showed to have very high expression of CD44v. For comparison, two androgen-independent cell lines were run: the moderately aggressive PC-3 cell line from which the above transformants were ultimately derived, and DU-145. Western blot analysis (Figure 6) showed CTR– cells to have markedly lowered CD44v expression compared to the other transfectants. CD44v was just slightly decreased in CT–. However, CD44s expression was increased in CTR– cells. Markedly slowed growth in the CTR– cells, with less in the CT– cells, were also observed.

Discussion

In this study, it was found that introduction of CD44s or full-length transcripts in prostate cancer (PC) caused CD44s re-expression and a decrease in CD44v. This re-expression of CD44s, like RNA interference against CD44v (16), decreased *in vitro* invasion. Cell adhesion studies were performed in benign prostatic epithelial cells and PC cell lines that represented three levels of CD44v expression and aggressiveness. The presence and aggressiveness of the PC correlated with an incremental switch in the predominant ligand from hyaluronan to fibronectin. The fibronectin adhesion was revealed to depend on CD44's hyaluronan-binding site, and re-expression of CD44s shifted binding back to hyaluronan.

We had observed by immunohistochemical staining, Western blot analysis, *in situ* hybridization and RT-PCR, that the CD44v7-v10 portion of the variant sequence was overexpressed in PC, while expression of CD44s and CD44v2-v6 isoforms was down-regulated (15, 16, 19). In PC, CD44v produced a 66 kDa immunoreactive fragment (15) owing to cleavage of the extracellular domain by membrane-associated metalloproteases (1, 2, 24, 25). Moreover, this

CD44v7-10 overexpression had functional significance; by silencing CD44v7-v10 expression through RNAi against CD44v9, invasiveness into a Matrigel artificial basement membrane was greatly reduced (16). A change to predominant CD44v expression may influence growth as well as invasion. In non-neoplastic cells, the intracellular domain (C-terminal) of intact CD44s also bound merlin resulting in a growth-inhibitory signal (24, 26). CD44's intracellular domain also bound actin, regulating cell shape (27) and tumor progression *via* cell signaling (28). These effects on the cytoskeleton may increase cell motility. Studies aimed at altering cell adhesion proteins in PC could also include MUC18 (CD146, or MelCAM). MUC18 is the only other protein that was increased in PC of all grades (21) and it conferred metastatic potential to PC in mice (29). We prioritized CD44v since our studies indicated that CD44v played a greater role than MUC18 in PC invasion (16).

Here, $G_s\alpha$-QL cell tumors were chosen for *in vivo* RNAi against CD44v because this cell line is the most aggressive with the highest CD44v expression of those we have studied (16), thus providing the opportunity to observe the most dramatic effects from RNAi. The cells were established (G.V.S) from metastatically capable PC-3 cells stably transfected to express the *gsp* mutant of the $G_s\alpha$ protein using the mammalian expression vector pcDNA3.1 (17). This mutant lacks GTPase activity and thus is constitutively active. The role of $G_s\alpha$ protein activity in PC invasion has been well-documented (16-18). PC transfected with mutant $G_s\alpha$ showed increased cAMP accumulation, increased DNA synthesis (17), faster proliferation (17), greater invasiveness *in vitro* and more metastasizing ability *in vivo* (18). We showed that increased $G_s\alpha$ protein activity correlated with expression of CD44v *in vitro* (16) and *in vivo* in mouse xenografts (19). Furthermore, calcitonin functions as an autocrine/paracrine factor in prostatic growth and its receptor is coupled to $G_s\alpha$. Calcitonin was able to stimulate PC invasion in four cell lines tested (19). This provided the first evidence for a link between an autocrine/paracrine factor, its receptor and signal transduction complex, the $G_s\alpha$-mediated raising of intracellular cAMP, and CD44 expression in PC. In non-small cell lung cancer, a similar phenomenon, mediated by intracellular cAMP, had been shown to increase CD44s (30).

For forced overexpression of full-length CD44 or CD44s, we chose the moderately invasive PC-3 cells because we were unsure whether overexpression of full-length CD44, by providing abundant normal CD44 mRNA, would be preferentially spliced to CD44s or to CD44v. Further, we did not know whether these changes would accelerate or decelerate aggressive properties, and we wanted to document either. In both overexpression transfectants, CD44s expression was restored, and this counteracted the expression of CD44v and its pro-invasive action in Matrigel

assays. The 1.2 kB CD44s DNA that we re-expressed came from prostatectomy specimens and was missing part of exon 18, similar to the frequent loss of exons or retention of introns in other cancers (7, 9). Part of exon 18 forms the transmembrane domain (16). This finding could account for the inability of the protein to insert in the membrane, hence loss of the intense membranous immunoreactivity in non-neoplastic prostate (10). Despite this defect, the CD44s still had significant effects on invasion and adhesion. Apparently, sufficient re-expression of CD44s can overwhelm the aberrant splicing of CD44 into the v7-10 variant that promotes invasion (16). These results were in accord with earlier reports that CD44s was a suppressor of cancer metastasis (3) and shed light on its mechanism of action.

CD44s re-expression restored hyaluronan binding but reduced fibronectin binding. First, reduced hyaluronan binding ability in PC was observed, with the reduction in binding being steepest in the more aggressive $G_s\alpha$-QL and PC-3 cells compared to LnCaP. An activating CD44 monoclonal antibody can induce hyaluronan binding only when monovalent antibody fragments are complexed (1, 2). Moreover, the inclusion of variant exons in the stem can alter the affinity of the oligomerized CD44 molecules for its ligand (2). Thus, CD44v's reduced oligomerization may explain why PC had negligible affinity for hyaluronan. Hyaluronan expression in PC was increased compared to normal and hyperplastic prostate (26, 31). This increase was associated with higher PC stage and grade (26, 31) and recurrence (32), but also with a decrease in CD44s (4). Simpson *et al.* showed that pericellular hylauronan and hyaluronan synthase expression were increased in aggressive PC3M-LN4 cells compared to LnCaP (33). Since the PC-specific CD44v appeared not to bind hyaluronan, and this CD44-hyaluronan interaction normally inhibits growth and migration (1, 2, 24, 25), therapy against aggressive PC that restored a benign, CD44s-predominant expression pattern could capitalize on this increased stromal hyaluronan. Thus, the reduced growth and invasion we observed in CD44s or CD44 full-length transformants may correlate with their stromal interactions, becoming more like those of indolent LnCaP cancer or benign prostate. Notably, this trend was opposite to that of colon cancer, where CD44s is overexpressed. Silencing of that expression reduced adhesion to hyaluronan and osteopontin (23). We disclosed significant osteopontin binding in benign prostate and, to a lesser extent, in LnCaP but not other tumor cell lines. This suggests that CD44s, and possibly CD44v, in the prostate bind osteopontin. Osteopontin production has been noted immunohistochemically in more than half of benign and malignant prostates (34), but our PC-3 cells overexpressing CD44s did not show restored binding to osteopontin.

A second notable finding from the adhesion assays was that CD44v had a special role in binding fibronectin. Several studies in other cancers support such a role in fibronectin

production or binding. Bates *et al.*, studying colon carcinoma cells, found their expression of CD44v to be accompanied by fibronectin secretion and expression of an integrin, with resulting anti-apoptosis and growth signals (35). In renal cell carcinoma, Brenner *et al.* found β_1-integrins, but not CD44, to be important for migration into fibronectin; however, they used an antibody that was not to the binding site of CD44s, thus not testing the binding site or the role of variant isoforms (36) which are overexpressed (4). Casey and Skubitz, using ovarian carcinoma cells, performed migration assays with or without antibody against the CD44 binding site or against β_1-integrin (37). The anti-integrin treatment almost totally inhibited migration towards fibronectin. However, anti-CD44 caused 60% inhibition of migration towards fibronectin, collagen type IV and laminin. Intact hyaluronan also inhibited interaction with these ligands. Like us, they postulated that fibronectin binding and migration uses an *integrin-dependent*, and a separate *integrin-independent* mechanism, the latter requiring the hyaluronan-binding site of CD44 (37).

We previously showed (19) that calcitonin, whether endogenous or exogenous, altered splicing in favor of CD44v. Moreover, our pharmacological manipulation of $G_s\alpha$ or its coupled, downstream enzyme, adenylyl cyclase, mimicked these effects. The linkage of a paracrine factor and its associated growth and cell signaling cascade to splicing of any cell adhesion molecule was a novel finding. Our current findings, that CT–, and especially CTR–, have a relative reversion to CD44s expression, add further support for an effect of calcitonin on splicing. The CT signaling pathway interacts with two candidate mitogenic, MAP (mitogen-activated protein) kinase pathways that have been postulated to cause alternative splicing of CD44 (38). One of these is the pathway involving MEK, which is part of the calcitonin receptor-coupled $G_s\alpha$ pathway, acting *via* cAMP which activates PKA. A direct effect of CT exerted on uroplasminogen activator (uPA) has been described (39). This signal is transmitted to uPA receptors, activating a pathway involving c-jun N-terminal kinase (JNK). The logical extension of our discovery, therefore, would be to use newly available pharmacological inhibitors against MEK and JNK (40) and to test their influence on CD44 splicing.

One notable observation was that CTR– had greater CD44v suppression than CT–. This finding suggests that calcitonin receptors might be activated by substrates other than calcitonin in the CT– cells, leading to splicing to produce relatively high CD44v levels. Without calcitonin receptor, however, the $G_s\alpha$ pathway and its downstream effects on MEK are blocked, arguing for the greater importance of the MEK pathway than the JNK pathway in CD44 splicing.

In conclusion, both the tissue-specific CD44v in PC and the loss of CD44s expression present targets for gene therapy to reverse growth and suppress metastasis. The mechanism for the functional effects of CD44v may have to do with alterations in the stromal ligands it binds compared with CD44s.

Acknowledgements

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