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Review Article

Cell adhesion molecule CD44: its functional roles in prostate cancer

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Abstract: CD44 is a cell adhesion glycoprotein that also governs cell signaling. Dysregulated CD44 expression characterizes most human cancers, including prostate cancer (PCa). PCa loses expression of CD44 standard (CD44s) that is present in benign epithelium, and overexpresses the novel splice variant (v) isoform, CD44v7-10. We studied CD44 in PCa for more than a decade, and in a series of papers, established its functional significance. Using retroviral gene delivery to PC-3M PCa cells, we expressed luciferase-only, enforced CD44s re-expression as a fusion protein with luciferase at its C-terminus or as a protein separate from luciferase, or knocked down CD44v7-10 by RNAi. Invasion, migration, proliferation, soft agar colony formation, adhesion, Docetaxel sensitivity, and xenograft growth assays were carried out. Compared to luciferase-only PC-3M cells, all 3 treatments reduced invasion and migration. Growth and soft agar colony formation were reduced only by re-expression of CD44s as a separate or fusion protein but not CD44v7-10 RNAi. Hyaluronan and osteopontin binding were greatly strengthened by CD44s expression as a separate protein, but not a fusion protein. CD44v7-10 RNAi in PC-3M cells caused marked sensitization to Docetaxel; the 2 CD44s re-expression approaches caused minimal sensitization. In limited numbers of mouse subcutaneous xenografts, all 3 alterations produced only nonsignificant trends toward slower growth compared with luciferase-only controls. In further work, we tested the effects of the anti-growth compound silibinin, a milk thistle derivative. Using a luciferase promoter construct to test for CD44 promoter activity, silibinin significantly and dose-dependently inhibited promoter activity at physiologic doses. Total CD44 RNA and CD44v7-10 RNA were significantly decreased; both were also decreased at the protein level. Phenyl-methylene hydantoins (PMH), guanidine alkaloids derived from Red Sea sponges, have the ability to increase cell-cell adhesion in prostate cancer cells and reduce invasion. Expression of CD44 total mRNA and CD44v7-10 were markedly decreased by PMH and its S-ethyl derivative. The oncogenic microRNAs, miR-373 and miR-520c, which interact with CD44, were studied in prostate cancer cells and human tissues. We found that they bound the 3' untranslated region of the CD44 RNA, and suppressed CD44 in prostate cancer, by preventing the translation of CD44 RNA, rather than by degrading the RNA. Thus, stable re-expression of CD44s reduces PCa growth and invasion in vitro, and possibly in vivo, suggesting CD44's potential as gene therapy. Finally, CD44v7-10 may be a target for chemosensitization, and plays a role in nutraceutical abrogation of tumor development. In vivo effects of CD44 alteration still need to be investigated by use of orthotopic or renal capsule xenografts, which confer a different stromal microenvironment than that of the subcutaneous grafts.

Keywords: CD44, prostate, splicing, standard, variant, silibinin, phenyl-methylene hydantoins, xenografts

Introduction

CD44, also known as Pgp-1, Ly-24, Hermes, lymphocyte homing receptor, H-CAM, and HUTCH-1, is a single pass transmembrane glycoprotein involved in cell-cell and cell-matrix adhesion and in cell signaling. It plays important roles in lymphocyte homing, inflammation, cell migration, signaling, and tumor metastasis [1,2]. Encoded by a gene located on the short arm of chromosome 11 at p13, CD44 consists of 20 exons over a length of about 60 Kb (**Figure 1**) [3,4]. CD44 proteins range in molecular weight from 85-230 kD for three reasons. First, a remarkable variety of mRNAs are generated by alternative splicing of ten variant exons in its pre-mRNA. Second, CD44 proteins undergo various degrees of post-translational modifications such as N- and O-linked glycosylation and glycosaminoglycanation. Third, they

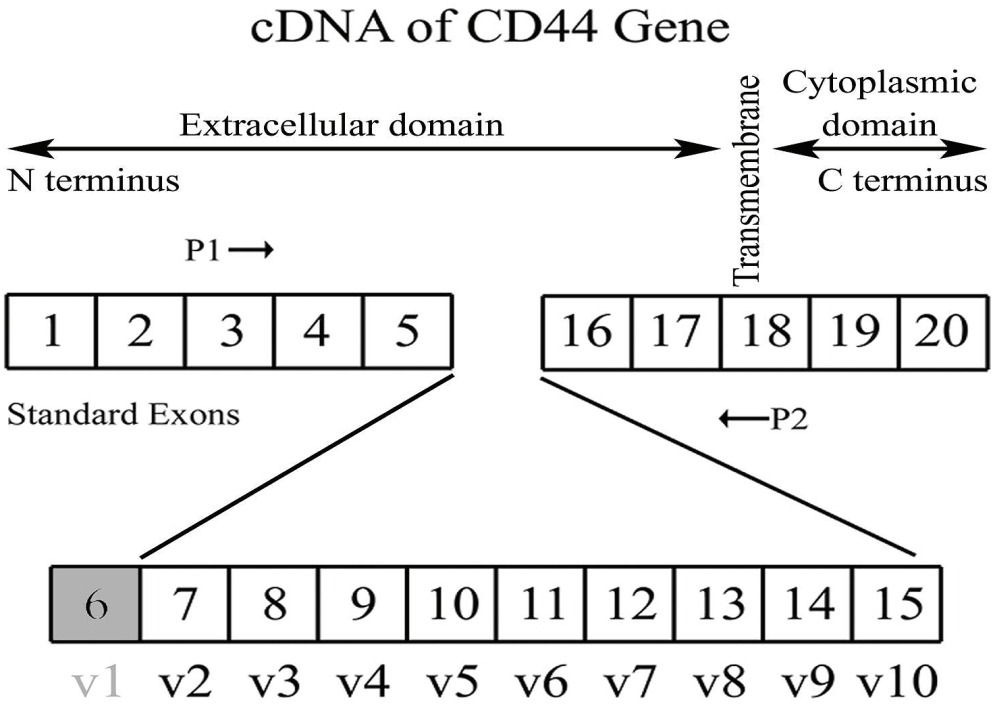

Figure 1. Schematic diagram of the structure of the CD44 gene. There are 10 standard exons, exons 1-5 and 16-20, that encode the ubiquitously expressed standard (CD44s) protein isoform. The intervening variant exons can be alternatively spliced in various combinations to encode variant protein isoforms. Exon 6, v1 of the human gene, contains a stop codon and does not get included in human CD44 mRNA, unlike in the mouse. Primers such as P1 and P2 can be used to amplify all the total (standard plus variant) mRNA. Exon-specific primers, such as to the v9 variant, can be used to quantitate the CD44v7-10 or other variants present.

often get shortened *in vivo*, owing to partial cleavage by matrix metalloproteases, and this effect is most pronounced in human cancer of all types [5]. The predominant form of CD44, designated hematopoietic or standard (**CD44s**) form, is translated to a polypeptide of 85-95 kD from exons 1-5 joined to exons 16-20 [6]. CD44 variant isoforms (**CD44v**) are the protein product resulting from inclusion of runs of alternatively spliced variant exons 6-15 (also referred to as exons v1-v10) that lengthen the protein's extracellular domain [7] and are implicated in metastasis [4,6]. CD44 spans the membrane, containing an extracellular domain, transmembrane domain, and cytoplasmic domain [1]. The N-terminal domain mediates the binding of total CD44 (standard plus variants) to the principal ligand hyaluronan [1], and also to extracellular matrix proteins such as osteopontin [8], collagen, laminin [9], and fibronectin [10]. The transmembrane domain is responsible for lymphocyte homing. The carboxyl cytoplasmic domain is anchored to the actin cytoskeleton [11] and to ezrin [12] and ankyrin [13] and is important not only in cell migration functions of CD44 but also in signal transduction [11].

CD44 in benign prostate

CD44 standard (CD44s) in the benign prostate, is a marker of basal cells [14-16], the stem cell compartment of the epithelium [17]. Both protein and mRNA studies have demonstrated predominant CD44s, expressed most strongly in basal cells and moderately in luminal cells in benign acini [16,18,19]. Immunoreactivity for certain variants, such as CD44v6 and also CD44v9, which is overexpressed in cancer, was confined to the basal layer in benign acini [16]. In other studies, uniform membranous staining of v3 and v6 isoforms was observed in the basal cells of the benign prostate, but less prominent staining occurred in the secretory cells [14,19]. Kallakury et al. showed that other variant isoforms (v4/v5, v7/v8, and v10) were focally expressed in benign acini [14].

CD44 in prostatic intraepithelial neoplasia

We found increased immunostaining in PIN for CD44v7 and v9 [16]. A loss of CD44s expression was seen in the basal and luminal cell layer of PIN lesions [16]. Moreover, lower intensity of CD44s, CD44v5 and CD44v6 were observed in high-grade PIN as compared to benign epithelium or low-grade PIN [19].

CD44 in primary prostate cancer

Notably, the functional effects of CD44 differ by tumor type, and CD44s is increased and has pro-invasive properties in some tumors such as colon cancer [20]. Prostate cancer, however, shows both downregulation of CD44s, and dysregulated splicing leading to increased, aberrant variant isoforms. CD44s was first described as decreased in proportion to tumor grade in 1996-8 [15,16,21]. However, subsequently our laboratory showed that patient-derived cancers had increased CD44v7 and v9 messenger RNA by *in situ* hybridization and RT-PCR [22]. By sequencing, this mRNA corresponded to the CD44v7-10 variants in most cases, or to part of CD44v6 plus v7-10 [18]. We achieved RNA interference against the expression of this sequence in cancer cell lines by targeting the CD44v9 epitope, and we significantly reduced Matrigel invasion by knocking down the variant, thus defining its functional role in invasion [18].

Subsequently, we used retroviral gene delivery to PC-3M cells to express luciferase-only, re-expressed CD44s as a fusion protein with luciferase at its C-terminus or as a protein separate from luciferase; or knocked down CD44v7-10 [23]. Invasion and migration were greatly reduced by all 3 treatments. Growth counts, and soft agar colony formation were reduced by the two or CD44s re-expression treatments only. The CD44v7-10 RNAi, however, caused marked chemosensitization to Docetaxel, whereas CD44s re-expression caused minimal but significant sensitization. In contrast to the marked efficacy of these 3 CD44 alterations *in vitro*, when we grew the altered cells as subcutaneous xenografts in limited numbers of nude mice (at least 7 per group), growth inhibitory effects were less. Pairwise comparisons of these 3 treatments showed only non-significant trends toward efficacy [23]. However, different or divergent effects between subcutaneous tumor cell grafts and *in vitro* effects have often been described for other cell adhesion molecules: for example, CEACAM [24]. We will need to use orthotopic or renal capsule xenografts, conferring a different stromal and matrix metalloprotease microenvironment, as a next step to resolve this paradox. Possibly, tumor in those sites will behave differently, validating CD44s and CD44v7-10 as clinical therapeutic targets.

Mechanisms of altered CD44

To elucidate the precise mechanism of CD44 down-regulation in malignancies, several studies were performed and showed that methylation and hypermethylation of CD44 gene promoter correlated with reduced total CD44 expression (which is mostly CD44s) and progression of prostate cancer [25-27]. Singal et al. studied methylation of the promoter of CD44 along with that of 6 other genes using methylation-specific RT-PCR in 81 prostatectomy specimens and 42 benign hyperplasia specimens [28]. In a large study of 90 cancers, CD44 promoter methylation was found in 33% of cases, which correlated with tumor grade but not stage [28]. Specimens from black men had more methylation than those from white men. More methylation was found in cancer. The degree of methylation was higher in stage III compared to stage II and in Gleason score ≥ 7 than Gleason score ≤ 6, but no multivariate analysis was done to assess independent predictive value.

But while methylation may influence total CD44 and CD44s, only aberrant splicing can explain the increased CD44v7-10 expression that characterizes cancer. We applied drug inhibitors against MEK, p38, and JNK, components of the MAP kinase pathway, to $G_s\alpha$-QL cells, which have high basal levels of CD44v7-10. We showed that the protein kinase A-MEK/ERK pathway, but not JNK, is required for total CD44 transcription. The p38 pathway, possibly through other downstream effectors, modulates CD44 splicing to yield mature CD44v7-10 mRNA [29].

Another influence on CD44v7-10 expression is paracrine calcitonin (CT), and possibly other growth factors, that stimulate it. Dr. Girish Shah and colleagues showed that prostate cancer cell growth and invasion are stimulated

by increased or constitutive CT expression. CT, acting through CT receptor and its effector, the $G_s\alpha$ protein, activates urokinase-type plasminogen activator (uPA), and the protein kinase A pathway [30]. Cells with constitutive CT expression have higher mRNA and protein levels of CD44v7-10 [18]. We found that preferential splicing toward CD44v7-10, which favors fibronectin binding, could be stimulated by calcitonin [31]. We first discovered that PC-3M cells that ectopically expressed wild type $G_s\alpha$-QL protein and were grown orthotopically in nude mice prostates, had increased CD44v9 mRNA and protein compared to parental PC-3M cells. Cells with mutant, constitutively active $G_s\alpha$-QL had still higher levels of CD44v9 [32]. The same was also true in LNCaP, PC-3, or their derivatives in response to exogenous CT, studied for CD44v9 by western blot [32]. By quantitative RT-PCR, CT+ cells, made to express CT endogenously, had five-fold more CD44 mRNA and more protein than parental PC-3M cells [29]. Moreover, in cells with CT knocked down called "CT-minus," exogenous 50 to 250 μM CT did not affect total CD44 RNA, but dose-dependently increased CD44 variant mRNA to five to seven-fold. This effect was not seen in cells with CT receptor knocked down, ruling out effects of CT not mediated by its receptor. The stimulation of CD44 variant protein was attenuated by cycloheximide, suggesting that *de novo* protein synthesis was required, and it was not simply a result of protein stabilization [29]. Moreover, CT's action on CD44 variant depended on an intact p38 pathway [29].

Mechanisms of CD44s suppression of metastasis

CD44s is significantly decreased in metastatic cancer compared to matched primary cancer [16,21]. Based on this discovery, Gao et al. were first to identify CD44s as a metastasis suppressor gene in prostate cancer. Using the Dunning system R-3327 rat prostatic cancer sublines, down regulation of CD44s expression both at the mRNA and protein levels correlated with increased metastatic potential. Concomitantly, they found that enhanced expression of CD44s in highly metastatic AT3.1 rat prostatic cells suppressed metastasis by more than 60% without affecting tumorigenicity. Additional studies demonstrated that metastasis suppression by standard CD44 isoform does not require the binding of prostate cancer cells to CD44's main ligand, hyaluronate [33]. It was later found that prostate cancer, in which CD44v7-10 predominates over CD44s, bound more strongly to fibronectin than to hyaluronan [31]. However, we succeeded in restoring a benign-like binding profile to cancer cells. That is, enforced CD44s overexpression [23] or CD44v7-10 knockdown [31] were both effective in restoring some hyaluronan binding or reduce fibronectin binding by PC-3M cells. Further work showed that neither the standard form nor any variant isoform of CD44 were expressed in metastatic prostate cancer [14,15,34].

Nutraceuticals and CD44

Our laboratory tested the effects of three nutritional supplements that are taken by those at high risk of prostate cancer and prostate cancer patients, or that show potential for that use. Recently there has been interest in the nutritional chemoprevention of prostate cancer by the anti-growth compound silibinin, a milk thistle derivative. Using a luciferase promoter construct to test for CD44 promoter activity, we found that silibinin significantly and dose-dependently inhibited promoter activity [35] at doses that are physiologic [36], suggesting a transcriptional site of action. Inhibition was up to 33% at the supra-physiologic dose of 200 μM. Correspondingly, total CD44 RNA was inhibited by 36%, with 42% inhibition of CD44v7-10. Both were also decreased at the protein level. As expected, adhesion assays demonstrated reduced adhesion to hyaluronan after dosing with silibinin [35]. The main problem with using silibinin clinically, however, was lack of penetration into prostate tissue, owing to silibinin's short half-life [36]. In concordance with this observation, prostate tissues from 6 men with prostate cancer treated with silibinin and 7 treated with placebo did not differ in immunoreactivity for CD44v9, the largest portion of the protein product resulting from inclusion of the CD44v7-10 exons [35].

Phenyl-methylene hydantoins (PMH), guanidine alkaloids derived from Red Sea sponges, have antifungal, antiviral, and other medicinal properties including increasing cell-cell adhesion in prostate cancer cells and reducing Matrigel invasion [37]. Because CD44 has functional significance in both of these phenomena, we decided to test the effect of synthetic PMH and S-ethyl PMH (S-PMH) synthesized by K. El-Sayed

and M. Mudit (University of Louisiana) on benign and malignant prostate cancer cells [38]. PMH and S-PMH markedly decreased expression of CD44 total mRNA and CD44v7-10. Both decreased total CD44 and CD44v7-10 protein, with S-PMH being somewhat effective. Adhesion assays demonstrated reduced adhesion to hyaluronan, a consequence of lowered CD44s, and to fibronectin, reflecting lower CD44v7-10 [38].

Vitamin D, known for its tumor suppressive effects on prostate cancer [39,40], has been studied pharmacologically by certain of our colleagues at the Univ. of Colorado [41]. We tested the effect of vitamin D and an analog on total CD44 expression and splice variant expression in G_sa-QL and ALVA-3 (a PC-3 derivative) cells. Effects of vitamin D or its butyl ester (BE) analog ($1\alpha,3ß,24(OH)_3$-22-ene-24-cyclopropyl-25-n-butyl-ester-vitamin D_3) at 10^{-7} or 10^{-8} M doses, or of the vitamin D receptor antagonist ZK159222 were tested for 24 hr. Our final conclusion was that there was no significant change in CD44s or CD44v levels. Using a luciferase promoter construct, it appeared that vitamin D did not alter CD44 transcription (unpublished data).

MicroRNAs and CD44 in Prostate Cancer

MicroRNA (miRNA) is a recently discovered class of noncoding RNA. miRNAs-373 and -520c in breast cancer cells, suppressed CD44s, and promoted breast tumor development, invasion, and metastasis according to Huang et al [42]. By western blot analysis, using antibody to total CD44, CD44 was decreased in cells after enforced expression of miR-373 or 520c. Moreover, they showed evidence of direct effects of miR-373 and 520c on breast cancer cells. Using shRNA against CD44 in these cells increased their migration whereas ectopic enforced expression of CD44 without the 3' untranslated region reduced migration. Use of antisense RNA against miR-373 (but not a mutated control) in MCF-7 cells reversed its inhibitory effect on CD44 as reported by a CD44 3'UTR luciferase plasmid [41].

We interrogated prostate cancer cells and tissues to determine whether miR-373 and miR-520c were oncomiRs. Expression of both was, surprisingly, decreased in PCa cells and tissues, in proportion to their decreases in total CD44 mRNA [43]. Exogenous miR-373 caused a dose-dependent increase in total CD44 RNA, and a decrease in CD44v7-10 RNA, with an optimal dose at 6 nM. At the protein level, however, both microRNAs suppressed CD44. Transwell migration and invasion were stimulated by miR-373 and miR-520c [43]. The microRNAs exhibited 3' UTR binding as assessed by a 3' UTR luciferase reporter construct but did not bind to CD44 promoter [43]. Thus, miR-373 and miR-520c exert an effect on CD44 in PCa by preventing the translation of CD44 RNA, rather than by degrading the RNA. Further data indicate that miR-373 and 520c transfection increase cell proliferation and confer resistance to a range of doses of the commonly used chemotherapy drug docetaxel. Although these microRNAs were decreased in PCa, their functional effect on prostate cancer cells was pro-tumorigenic: pro-growth and pro-invasive [43].

These two microRNAs had been shown to function as oncomiRs in breast cancer [42], by causing CD44 RNA degradation. Our findings, conversely, suggested CD44 RNA accumulation with translational repression was the mechanism by which they suppress total CD44 in the prostate [43], which is mostly CD44s. miR-373 and miR-520c, according to our work, do function as oncomiRs in prostate cancer, by binding the 3′ untranslated region (UTR) of CD44 mRNA, suppressing its translation, and promoting cancer cell migration and invasion [43].

Conclusions

Our several CD44 papers established its functional significance in prostate cancer. CD44 expression and splicing in cells could be regulated by paracrine calcitonin, two oncogenic microRNAs and nutraceuticals, and CD44v7-10 conferred chemoresistance. These are some of the many facets of CD44's pivotal role in tumor development. The next logical step toward human gene therapy will be to pursue *in vivo* mice experiments that use orthotopic or renal capsule xenografts, which confer a different stromal microenvironment than that of the subcutaneous grafts.

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