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Original Article

Phenyl-methylene hydantoins alter CD44-specific ligand binding of benign and malignant prostate cells and suppress CD44 isoform expression

Kui Yang, Yaqiong Tang, Kenneth A. Iczkowski

Department of Pathology, University of Colorado Denver Health Science Center, Aurora, Colorado, USA.

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Abstract: Dysregulated CD44 expression is a feature of most human cancers, including prostate cancer (PCa). PCa loses expression of CD44 standard (CD44s) which is present in benign epithelium, and overexpresses a novel splice variant isoform, CD44v7-10, specifically facilitating fibronectin binding and invasion. Naturally-occurring or synthetic phenyl-methylene hydantoin (PMH) and S-ethyl PMH (S-PMH) can reportedly augment cell-cell adhesion, and reduce invasion and growth of PCa. Benign BPH-1 and malignant PC-3M prostate cells were treated with PMH or S-PMH for 36 h and cells were harvested. Cell adhesion assays were carried out. Cancer cells' expression of total CD44 and CD44v7-10 were tested by western blot analysis and real-time RT-PCR. Compared to BPH-1 or PC-3M cells treated with vehicle only, PMH-or S-PMH-treated benign and malignant cells had decreased adhesion to hyaluronan ($p=0.001$ to 0.007) and fibronectin ($p<0.001$ to 0.047). Both compounds decreased PCa expression of CD44 total mRNA (representing mostly CD44s, to 0.076±0.033 and 0.254±0.123 of control) and CD44v7-10 (to 0.386±0.279 and 0.115±0.037 of control). S-PMH but not PMH decreased CD44 total protein, while both decreased CD44v7-10 protein. Both hydantoins lowered β-catenin, as reported previously. Both only slightly decreased β1-integrin, the definitive receptor for fibronectin. In conclusion, the ability of PMH and S-PMH to decrease hyaluronan adhesion appears to be mediated through decreased CD44s, while the decrease in fibronectin adhesion correlates with, and may be mediated by, decreased CD44v7-10.

Keywords: Alternate splicing, CD44, hydantoin, phenyl-methylene hydantoin, prostate cancer, hyaluronan, fibronectin

Introduction

About 30% of cases of prostate cancer (PCa) undergo transition from quiescent to aggressive. In this transition, altered splicing of the hyaluronan receptor CD44 occurs that allows tumor cells to detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels. CD44 is a transmembrane molecule encoded by an alternately spliced gene. The standard (**CD44s**) isoform is ubiquitous, but inclusion of one or more of 10 variant (**v**) exons lengthens the extracellular stem, producing tissue-specific (**CD44v**) isoforms. CD44 is involved in multiple cellular functions. Its N-terminus enables cell-cell adhesion and binds hyaluronan and other matrix ligands, while the C-terminus links the cell's membrane to actin and ankyrin in the cytoskeleton, modulating shape and motility.

In prior work, we isolated RNA from clinical PCa tissues and discovered that expression of CD44v7-10 variant isoform constitutes a unique PCa signature, consistently expressed in primary and metastatic PCa [1], and most strongly expressed in androgen-independent PCa cell lines [2-3]. RNA interference against CD44v7-10 caused a 69% reduction in inva-

sion index compared to untreated control cells [3] and altered ligand-binding affinities [4]. Moreover, benign prostate strongly expresses CD44s and CD44v6, while PCa loses ability to splice pre-mRNA into CD44s [3, 5, 6], or certain variants other than CD44v7-10 [1,7]. CD44v7-10 was important for the increased fibronectin binding of cancer cells [4].

Recently, Mudit et al. [8] characterized guanidine alkaloid compounds derived from Red Sea sponge *Hemimycale arabica*, that have antitumor, antiviral, antifungal, and anticonvulsant properties. Potent anti-growth and anti-invasive properties were noted against PC-3M prostate cells. Shah et al. [9] reported that phenyl-methylene hydantoin (PMH) and its S-ethyl derivative, S-ethyl PMH (S-PMH)—equally potent whether extracted from sponges or synthesized—could augment cell-cell adhesion, and reduce Matrigel invasion, including invasion stimulated by calcitonin which functions as a pro-invasive paracrine hormone. PMH compounds could also reduce spheroid disaggregation in an *in vitro* assay that measures metastatic potential of tumor cells, and significantly inhibit growth and metastasis of PCa in orthotopic tumor xenografts [9]. Since CD44 is a key player in PCa invasion, we tested PMH compounds' effects on adhesion to known ligands of CD44 and on total and variant CD44 expression.

Materials and methods

Cell lines

Benign BPH-1 prostate cells were from American Type Culture Collection (Manassas, VA). PC-3M cells, a metastasis-derived variant of PC-3, were from Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. The culture medium for PC-3M cells was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) and antibiotics. Cells overexpressing CD44s as a protein "Separate" from luciferase, were made by infecting PC-3M with virus packaged in 293 cells after inserting CD44s-RSV promoter-luciferase sequence behind the cytomegalovirus promoter of Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), and selecting through its puromycin resistance gene.

For cell set-up, cells in a flask were trypsinized, medium with serum was added to neutralize trypsin, and cells were stained in Trypan blue and counted by grid method [4]. Cells were treated with 50 μmol/L of synthetic PMH or S-PMH (generous gifts of Dr. Girish V. Shah, Univ. of Louisiana-Monroe) in DMSO, since this dose was most effective for enhancement of tight junction and adherens junction function, and for attenuation of calcitonin-stimulated invasion [9].

Cellular adhesion assays

At least 2 repeat assays were carried out [10] using trypsinized confluent untreated or virally treated cells. Each test condition was set in 5 wells and each experiment repeated with similar results. 96-well black-edged clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with optimal concentrations of ligands [10] using 8 wells to test each one, at 37°C overnight. As controls, 8 wells were coated with 1 mg/ml BSA to measure baseline nonspecific binding. $1x10^6$ cells suspended in 1 ml PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 min at 37°C. After two washes of the cells with PBS, cells with serum-free basal medium were added to plates at a density of $3x10^4$ per well and incubated at 37°C for 90 min. Fluorescence intensities at 530 nm were measured using a Bio-Tek FL-600 plate reader. Nonadherent cells were removed with 2 PBS washes. Fluorescence intensities with PBS in the wells were measured. Adhesion was calculated [10] as % cells bound=(100) fluorescence intensity post-wash / fluorescence intensity of total cells plated.

Real Time Quantitative RT-PCR

To confirm altered CD44 expression, we used a primer + probe set that detects CD44s, or a set that detects CD44v7-10 (Applied Biosystems, Foster City, CA) as we described [3,11]. Detection of 18S ribosomal RNA was done simultaneously as a normalizer. TaqMan data were analyzed by the $2^{(-\Delta\Delta C_T)}$ method [12] to determine fold change in gene expression (untreated cells=1.00). The ΔC_T was taken as the difference between the CD44v7-10 or CD44 total and the 18S ribosomal RNA C_Ts. The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of untreated cells as calibrator. Mean± standard deviation of normalized amount of RNA was calculated based on triplicates.

Western blot analysis

Cultured cells were directly lysed in dishes using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to the Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 µl of Kaleidoscope protein marker (Bio-Rad, Hercules, CA) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Mouse monoclonal antibodies were all used at 1 µg/mL including CD44v7/8 antibody (Bender MedSystems, Burlingame, CA) to assess CD44v7-10; CD44 total (standard + variant, Bender, 156-3C11), CD44s (LabVision, Fremont, CA), rabbit phosopho-merlin (Cell Signaling), β1 integrin (R&D, Minneapolis, MN), or β-catenin. Anti-β-actin antibody (Sigma, St. Louis) was used at a dilution of 1:5,000. Membranes were washed 3 x 10 min in TBS with 0.1% Tween-20 (TBST) and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) or 1:1,250 goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) was added in 5% skim milk for 1 h. After washing membrane with 1x TBST, reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Statistical Analyses

For all *in vitro* assays, data were expressed as mean ±SD. The significance of differences among group means was tested by two-tailed paired Student *t*-test. Statistical significance was set at $p<0.05$.

Results

Cell adhesion assay

PMH or S-PMH treatments reduced the adhesion of BPH-1 and PC-3M cells to both hyaluronan ($p=0.001$ to 0.007) and fibronectin ($p<0.001$ to 0.047) by one-third to one-half (**Figure 1**) compared to DMSO-only controls.

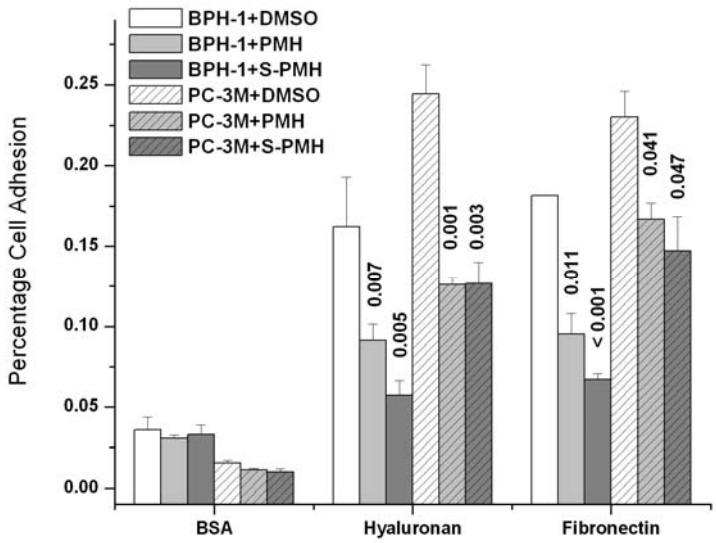

Figure 1. Cell adhesion assays. p values indicated above bars. In both benign (BPH-1) and cancer (PC-3M) cells, 50 µM PMH or S-PMH significantly reduced binding to hyaluronan and fibronectin compared to DMSO-only treated control cells. Baseline binding was assessed by coating plates with bovine serum albumin (BSA).

PMH and S-PMH effect on total CD44, phospho-merlin, and β-catenin

Western blot analyses in PC-3M cells showed that S-PMH, but not PMH, decreased total CD44 (**Figure 2**). Also, the phosphorylated, pro-growth form of merlin, a downstream mediator of the effects of CD44 signaling on proliferation, was decreased. Using an antibody to β-catenin, we duplicated the observation of Shah et al. [9] that both hydantoins decreased β-catenin. Real Time RT-PCR was used to demonstrate relative amounts of CD44 total mRNA (representing mostly CD44s). mRNA was decreased by PMH to 0.076±0.033, and by S-PMH to 0.254±0.123 of control.

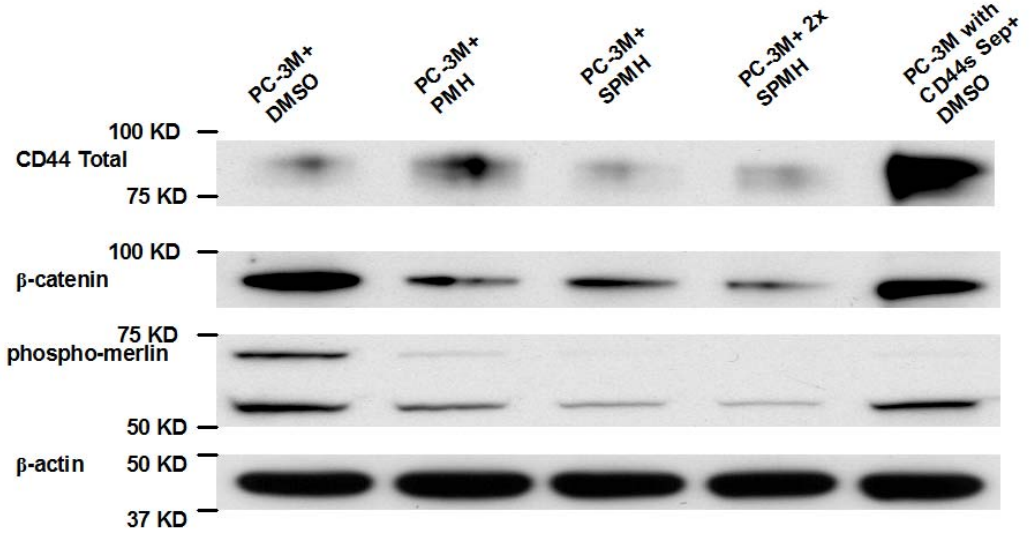

Figure 2. Detection in PC-3M prostate cancer cells of total CD44, CD44's intracellular effector phospho-merlin, and β-catenin, a subunit of the cadherin protein complex, as influenced by PMH or S-PMH. In lane 5, the CD44s-Sep cells were designed by us to overexpress CD44s and luciferase as separate proteins (Yang et al., unpublished observations), providing a positive control for high total CD44.

PMH and S-PMH effect on CD44v7-10 and β1-integrin

The definitive ligand for fibronectin is β1-integrin, particularly as part of integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$. To determine whether the altered fibronectin adhesion in the presence of the hydantoins was the result of abatement of high CD44v7-10 expression versus β1-integrin (CD29), western blot analyses were performed for both (**Figure 3**). PMH and S-PMH virtually abolished CD44v7/8-reactive protein, representing CD44v7-10, with only minimal effect on β1-integrin. The re-expression of CD44s somewhat suppressed CD44v7-10, consistent with what we showed previously [4]. By Real Time RT-PCR, CD44v7-10 mRNA after PMH was 0.386±0.279 of control and after S-PMH was 0.115±0.037 of control. Together, these results implicate suppression of splice isoform CD44v7-10 in the reduced fibronectin binding.

Although this study's emphasis is cancer cells, protein preparations of benign BPH-1 cells were also tested by western blot analysis for CD44v7/8, showing moderate inhibitions by PMH and S-PMH, and for total CD44, showing no change (**Figure 4**).

Discussion

We have shown that in aggressive PC-3M PCa cells, and benign BPH-1 cells, phenyl-methylene hydantoin (PMH) and S-ethyl PMH (S-PMH) significantly alter CD44 expression and cell adhesion to ligands that are known binding partners of CD44 isoforms. Overall, both seemed equally potent, although in prior work, S-PMH was 3 times more potent than PMH in inhibiting PC-3M cell spheroid dispersal, and more effective at extending mouse survival and preventing metastases [9].

CD44s, according to Miyake et al. [13], is the sole ligand in PCa that binds hyaluronan. The introduction of CD44s in PC-3 cells markedly enhanced the binding and migration of these cells to hyaluronan, but not to other extracellular matrix molecules. Thus, a reduced binding to hyaluronan correlates with the treatment effect of less total CD44 RNA and protein. CD44s is considered a metastasis suppressor in PCa. However, we noted that lymph node metastases of PCa seemed to re-express CD44s [1], and CD44 positivity is associated with basal cell/ stem phenotypes and high proliferation in prostate [14] and breast [15] cancers. Thus a reduction of total CD44, consisting mostly of CD44s, could partly explain

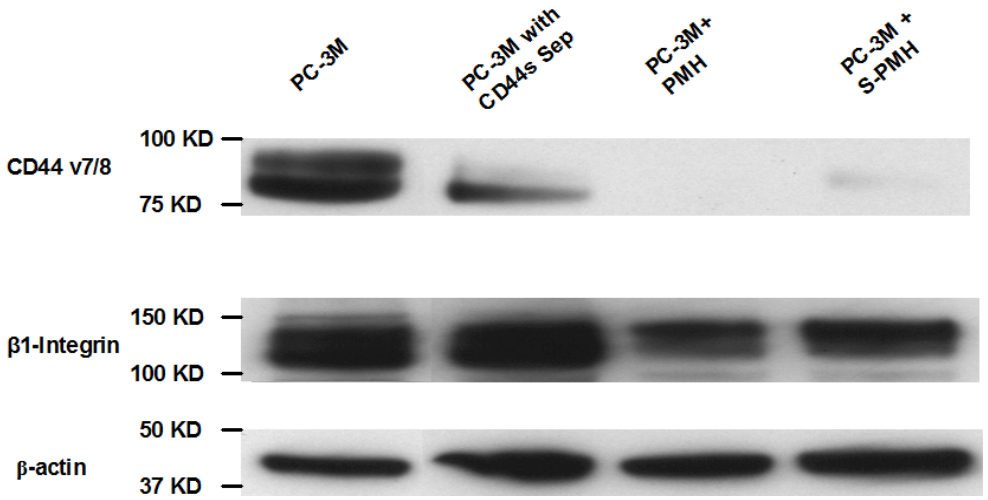

Figure 3. PC-3M prostate cancer cells showing alterations in CD44v7/8 and β1-integrin due to PMH or S-PMH. Detection at 85 kD of a cleaved form of CD44v7/8, a proxy for CD44v7-10, although decreased by overexpression of CD44s (lane 2), is almost abolished by PMH and S-PMH (lanes 3-4). β1-integrin is only slightly decreased by the compounds.

the antimetastatic potential [9] of PMH and S-PMH.

Fibronectin facilitates cell attachment via α4β1, αVβ3, and α5β1 integrins, with the latter being decreased in some cancers, but is also a ligand for CD44v. We showed previously that the predominant CD44v7-10 in PCa cells specifically binds to fibronectin [4]. The ability of the tested hydantoins to reduce fibronectin binding appears primarily attributable to altered CD44v7-10 expression (detected by anti-CD44v7/8), although β1-integrin expression minimally changed in their presence. Interestingly, PMH caused increased cell-cell adhesion of PC-3M cells, measured by the transepithelial resistance method [9]—standing in contrast to the decreased cell-extracellular matrix adhesion to these two important ligands reported herein.

Like Shah et al. [9], we performed western blot analysis for E-cadherin, and obtained no signal. E-cadherin was detectable by them only in the insoluble fraction (cell pellet), unless cells were stimulated by calcitonin,

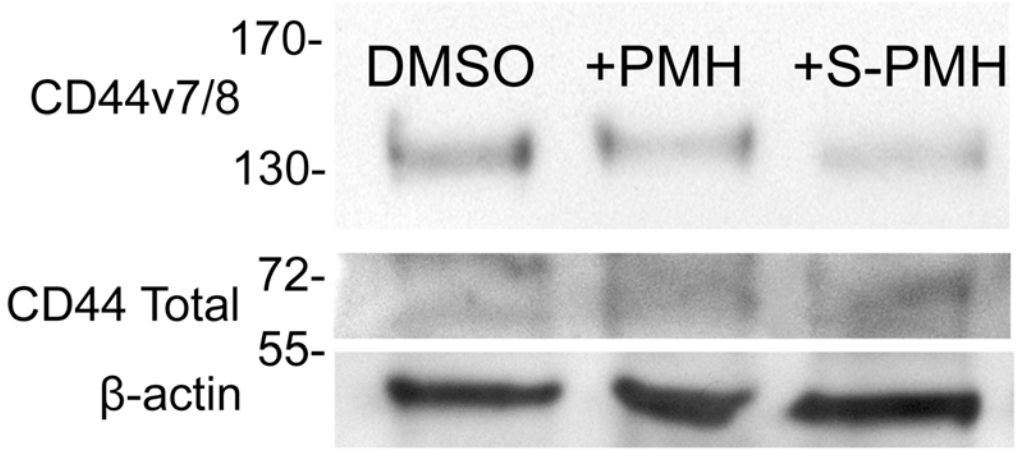

Figure 4. Benign BPH-1 cells were treated with DMSO, PMH, or S-PMH. Total CD44 is not perceptibly altered, while PMH causes a mild decrease in CD44v7/8 (lane 2), and S-PMH causes a greater decrease (lane 3).

thus our use of the soluble proteins for our studies gave the expected result. As in Shah et al., β-catenin was decreased by treatment with PMH, and although they did not study S-PMH, we found S-PMH to exert the same effect.

Given that metastasis suppression by CD44s is not entirely dependent on hyaluronan [16], the intracellular binding partners of CD44 may be implicated. We examined merlin, an intracellular protein that mediates the action of CD44 in a phosphorylation-dependent manner. Merlin is activated by phosphorylation under growth-promoting conditions and dephosphorylated under growth-inhibiting conditions including CD44-hyaluronan binding which inactivates it and circularizes it [17, 18]. Using antibody to phospho-merlin, we detected PMH- and S-PMH-induced decreases in both a hyperphosphorylated and a lower molecular weight, hypophosphorylated form of merlin. Since P-merlin is considered growth-promoting, this result suggests that merlin dephosphorylation plays a role in the growth-inhibitory effects of these hydantoins, perhaps orchestrated by less CD44s and less hyaluronan binding. In benign BPH-1 cells, the same trend was evident, consistent with Horiguchi et al. [19] who found that silencing of CD44 dephosphorylates merlin in benign cells.

The hydantoin compounds PMH and S-PMH are known to have antitumor, antiviral, antifungal, and anticonvulsant effects, reviewed by Mudit et al. [8], can be cost-effectively synthesized, and show minimal side effects in mice [9]. More recent investigations have found hydantoin derivatives also effective in inhibiting angiogenesis *in vitro* from cultured endothelial cells and inhibiting secretion of VEGF by osteosarcoma cells [20]. Here we show that both PMH and S-PMH reduce CD44 total and CD44 v7-10 expression in PC-3M cells, of which the latter isoform facilitates invasion [3]. These two changes, respectively, correlate with reduced adhesion to hyaluronan and fibronectin, and are likely to relate to the compounds' ability to inhibit PC-3M cell invasiveness [9].

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Please address correspondences to: Kenneth A. Iczkowski, MD, Pathology—Campus Mail Stop 8104, 12800 E. 19th Avenue, Aurora, CO 80045. Tel: 303-724-0155, Fax: 303-724-3712, E-mail: Kenneth.Iczkowski@UCDenver.edu

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