Advances in Brief

Metastasis Suppression by the Standard CD44 Isoform Does Not Require the Binding of Prostate Cancer Cells to Hyaluronate[1]

Allen C. Gao, Wei Lou, Jonathan P. Sleeman, and John T. Isaacs[2]

The Johns Hopkins Oncology Center, James Buchanan Brady Urological Institute, Department of Urology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231-1001 [A. C. G., W. L., J. T. I.], and Forschungszentrum Karlsruhe, Institute of Genetics, Karlsruhe D-76021, Germany [J. P. S.]

Abstract

Previous studies from this laboratory have demonstrated that down-regulation of the standard CD44 isoform at the mRNA and protein level is associated with the acquisition of high metastatic ability within the Dunning R-3327 system of rat prostate cancers. Additional studies demonstrated that transfection-induced enhanced expression of the standard CD44 isoform suppresses the metastatic ability of the AT3.1 Dunning subline without suppressing tumorigenicity. The standard CD44 isoform is a major cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronate. In this study, an investigation was made to resolve whether the ability of the standard CD44 isoform to suppress metastasis of the AT3.1 prostate cancer cells critically requires enhanced hyaluronate binding. Highly metastatic Dunning AT3.1 rat prostate cancer cells were transfected with expression plasmids encoding either the wild-type or mutant standard CD44 isoform. The mutant standard CD44 isoform construct encoded a protein unable to bind to hyaluronate. Transfectants were isolated and characterized with regard to their level of standard CD44 isoform expression, hyaluronate binding, tumorigenicity, and metastatic ability. Expression of the wild-type standard CD44 isoform increased the hyaluronate binding of prostate cancer cells and suppressed their metastatic ability without suppressing their tumorigenicity. Expression of the mutant CD44 standard isoform did not increase hyaluronate binding; however, it equally suppressed the metastatic ability of the AT3.1 prostate cancer cells. These results demonstrate that the metastasis suppression by the standard CD44 isoform is independent of its ability to bind to hyaluronate.

Introduction

CD44, also known as Pgp-1, Ly-24, Hermes, lymphocyte homing receptor, H-CAM, and HUTCH-1, is an integral membrane phosphoprotein that serves as a multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions (1). At least 20 isoforms can be generated by alternative splicing of the 20 exons within the 60-kb *CD44* gene (2). Besides alternative splicing, variations in *N*-glycosylation, *O*-glycosylation, and glycosaminoglycanation can occur, resulting in CD44 protein ranging from 85–230 kDa (2). The standard CD44 isoform that lacks the entire variable regions is a single-chain (85–95 kDa) molecule composed of a distal extracellular domain, a membrane proximal region, a transmembrane-spanning domain, and a cytoplasmic tail (2). A common characteristic of all of the CD44 isoforms is their ability to bind to extracellular matrix-associated hyaluronate with high affinity (3). However, the ability of CD44 molecules to bind to hyaluronate is strictly regulated and may need to be activated to bind to hyaluronate (4).

In several animal models, transgenic overexpression of specific

variant CD44 isoforms by tumor cells resulted in enhanced metastatic potential (5, 6), whereas other studies have demonstrated that repressed expression of the standard CD44 isoform is associated with malignant transformation of several tumor types including squamo- cells (7) and prostate tumors (8–11). Additional studies have demonstrated that overexpression of the standard CD44 isoform in colon carcinoma results in reduced tumorigenicity (12). Within the Dunning system of rat prostate cancer (8) and in human prostate cancers (9–11), down-regulation in the expression of the mRNA and protein of the standard CD44 isoform is associated with the acquisition of metastatic ability. Furthermore, transfection-induced enhanced expression of the standard CD44 isoform in the highly metastatic Dunning AT3.1 rat prostate cancer cells greatly suppresses their metastatic ability without suppressing their tumorigenicity (8).

The mechanism by which the standard CD44 isoform suppresses metastasis remains unclear. It is controversial whether hyaluronate, the major ligand for CD44, mediates this process. It has been demonstrated that hyaluronate is directly implicated in the regulation of tumor development of human melanoma (13) and the metastatic behavior of mouse melanoma (14), whereas in other reports, hyaluronate is not involved in the metastasis of lymphoma cells (15) and pancreatic carcinoma cells (16). In the present study, the question of whether enhanced hyaluronate binding induced by overexpression of the standard CD44 isoform is required for suppression of the metastatic ability of the Dunning AT3.1 rat prostate cancer cells was tested.

Materials and Methods

Cell Culture. AT3.1, a highly metastatic Dunning rat prostate cancer cell line, was characterized and maintained as described previously (17).

Expression Plasmids for Wild-Type and Mutant Standard CD44 Isoforms. The standard rat CD44 isoform expression construct used in this study has already been described (5). To create the standard rat CD44 isoform hyaluronate-binding mutant expression construct, the previously described CD44v4-v7 (R44→A) construct (18) was digested with *Ava*I and *Bgl*II to remove the v4-v7 domain and part of the constant domain but leave the mutant hyaluronate-binding site in the vector. The corresponding *Ava*I/*Bgl*II fragment of the standard CD44 was then ligated into the *Ava*I/*Bgl*II sites of the cleaved CD44v4-v7 R44→A vector to create the standard CD44 hyaluronate-binding mutant plasmid.

Transfection. The wild-type and mutant standard rat CD44 expression plasmids were transfected into AT3.1 cells by LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Transfectant clones were isolated and maintained under G418 selection as described previously (8).

Western Blot Analysis. Protein extract (60 μg) was subjected to 6% SDS-PAGE and electroblotted as described previously (8). The membranes were rinsed and incubated with 2 μg/ml antirat CD44 monoclonal antibody (BioSource International, Camarillo, CA) as described previously (8). Protein was detected using an enhanced chemiluminescence reagent as described by the manufacturer's instructions (Amersham, Arlington Heights, IL).

Hyaluronate-binding Assays. Immobilized hyaluronate-binding assays were performed as described by Sleeman *et al.* (16). Briefly, Coulter Counter

Fig. 1. Expression of the standard rat CD44 isoform protein in mutant standard CD44 clones (*CD44s-HA1*, *CD44s-HA2*, and *CD44s-HA3*), wild-type standard CD44 clones (*CD44s-1*, *CD44s-2*, and *CD44s-3*), AT3.1 parental cells, and neo control as assessed by Western blot analysis using antirat CD44 antibody. Sixty μg each of protein were subjected to 6% SDS-PAGE as described in "Materials and Methods."
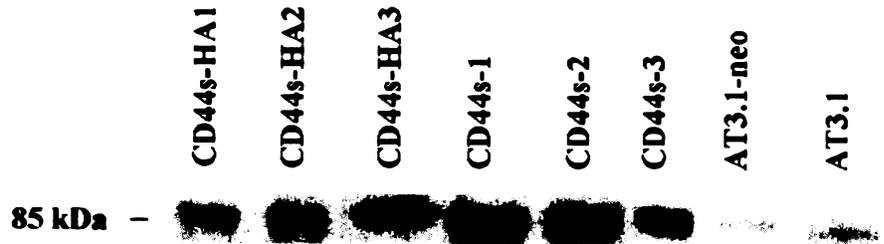

CD44s-HA1   CD44s-HA2   CD44s-HA3   CD44s-1   CD44s-2   CD44s-3   AT3.1-neo   AT3.1

85 kDa —

Table 1 *In vitro characteristics of parental AT3.1, neo, wild-type CD44s, and mutant CD44s transfectants*

Clone	Standard CD44 protein[a]	Hyaluronate-dependent plate binding[b]
AT3.1	0.2	45 ± 9
AT3.1-neo	0.2	43 ± 7
AT3.1-CD44s-1	5	72 ± 10
AT3.1-CD44s-2	12	75 ± 6
AT3.1-CD44s-3	10	79 ± 9
AT3.1-CD44s-HA1	5	50 ± 11
AT3.1-CD44s-HA2	8	48 ± 8
AT3.1-CD44s-HA3	9	52 ± 8

[a] The value for AT3.1-CD44s-3 was standardized to 10, and the values for the other clones were adjusted accordingly.

[b] Hyaluronate binding of the cell clones is expressed as the percentage of the mean cells bound *versus* the total number of cells inoculated. Hyaluronate-independent binding is <1%.

cups were incubated overnight with 0.5 ml of 1 mg/ml hyaluronate (Sigma) in PBS or with PBS alone for controls. Confluent cells were harvested, counted, and radiolabeled by incubation with L-[^{35}S]methionine in methionine-free RPMI 1640. After washing with PBS, 5×10^5 cells were added to each Coulter Counter cup and incubated for 30 min. Bound cells were lysed after washing, and the number of cells bound was calculated from the quantification of radiolabel in the lysate. Each sample was performed in triplicate.

Characterization of *in Vivo* Growth and Metastasis. To evaluate the *in vivo* growth rate and metastatic ability of the transfectant clones, 5-week-old male athymic nude mice (Charles River Laboratories, Frederick, MD) received s.c. injections in the flank with 1×10^5 cells. Thirty-six days later, the tumor-bearing animals were sacrificed. The tumors were weighed, and the lungs were inflated with Bouin's solution. Lung metastases were scored under a dissecting microscope.

Statistical Analysis. Values are expressed as the mean ± SE. Statistical analysis of significance was calculated by one-way ANOVA followed by the Newman-Keuls test for multiple comparison, with $P < 0.05$ considered significant.

Results

Generation of AT3.1 Transfectants Expressing either Wild-Type or Mutant Standard CD44 Isoform Protein. To test whether standard rat CD44 isoform has a similar effect on metastatic ability as the standard human CD44 isoform and whether hyaluronate, the major ligand of CD44, is involved in this process, expression vector constructs encoding either a wild-type standard rat CD44 isoform or a mutant standard rat CD44 isoform unable to bind to hyaluronate were generated. Each of these constructs was cotransfected with pSV2Neo into AT3.1 cells. Stable AT3.1 transfectants expressing either wild-type standard rat CD44 protein or mutant rat CD44 protein were selected. Expression of the standard rat CD44 protein was tested by Western blot analysis with antirat CD44 standard monoclonal antibodies, as described in "Materials and Methods." Three clones from each construct were retained for additional experiments. As shown in Fig. 1, representative clones transfected with either the wild-type standard rat CD44 isoform or the mutant standard rat CD44 isoform expressed rat CD44 protein with a molecular mass of 85 kDa. Furthermore, the levels of CD44 expression of the mutant standard CD44

clones were comparable to those of the wild-type standard CD44 clones.

Hyaluronate is a major ligand of activated CD44 molecules. Therefore, the effect that ectopic expression of wild-type standard rat CD44 as well as mutant standard rat CD44 by highly metastatic AT3.1 prostate cancer cells had on the ability of the cells to bind to hyaluronate was tested. The wild-type standard rat CD44-expressing cells displayed a higher binding to hyaluronate-coated surfaces than did parental AT3.1 cells, whereas mutant standard rat CD44-expressing cells did not display an increase in their capacity to attach to hyaluronate-coated surfaces compared with that of parental AT3.1 cells (Table 1). These results demonstrate that although the mutant standard rat CD44-expressing cells expressed similar amounts of ectopic standard rat CD44 protein as wild-type standard rat CD44-expressing cells, their ability to bind to hyaluronate was not affected as compared with that of parental AT3.1 cells, due to the single R44→A point mutation in the transfected mutant rat CD44 cDNA.

Effect of Ectopic Expression of Wild-Type Standard Rat CD44 or Mutant Rat CD44 on AT3.1 Metastatic Ability. Several independent transfectant clones from either wild-type standard rat CD44 or mutant rat CD44, along with parental AT3.1 and AT3.1-neo control, were implanted s.c. into the flanks of nude mice, respectively. All animals injected with the wild-type standard rat CD44-expressing cells showed a more than 95% suppression of the number of lung metastases/mouse ($P < 0.05$) without inhibition of the growth of the primary tumors (Table 2). Interestingly, metastasis suppression of the AT3.1 rat prostate cancer cells induced by overexpression of standard rat CD44 is even more profound than the suppression induced by overexpression of standard human CD44, as described previously (8). Like the wild-type standard rat CD44 isoform, the mutant rat CD44-expressing cells displayed more than 90% suppression of the metastatic ability to the lungs without suppressing tumorigenicity (Table 2). In contrast, no metastasis suppression was observed in the neo control transfectant cells (Table 2).

Discussion

The standard CD44 isoform is normally expressed on the plasma membrane of human prostate glandular cells (9–11). The standard

Table 2 *In vivo metastatic ability of parental AT3.1, neo, wild-type CD44s, and mutant CD44s transfectants*

Clone	Metastatic ability[a] (no. of lung metastases/animal)	Tumor weight (g) at excision[a]
AT3.1	72 ± 10 (4)[b]	7.5 ± 1.3
AT3.1-neo	68 ± 11 (4)	7.2 ± 1.2
AT3.1-CD44s-1	2 ± 3 (5)[c]	6.8 ± 1.5
AT3.1-CD44s-2	0 (5)[c]	6.2 ± 1.2
AT3.1-CD44s-3	0 (5)[c]	7.9 ± 1.3
AT3.1-CD44s-HA1	3 ± 2 (4)[c]	7.2 ± 1.5
AT3.1-CD44s-HA2	0 (5)[c]	6.7 ± 1.8
AT3.1-CD44s-HA3	5 ± 4 (5)[c]	7.4 ± 1.7

[a] Mean ± SE at 36 days after inoculation of 1×10^5 cells.

[b] Numbers in parentheses, number of animals/group.

[c] $P < 0.05$ compared to the mean number of metastases with AT3.1-neo transfectant.

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CD44 isoform expression is down-regulated at both the mRNA and protein levels during human prostate cancer progression, with down-regulation being correlated with higher tumor grade, aneuploidy, and distant metastasis (9–11). Additional studies by Noordzij et al. (10) demonstrated that the loss of standard CD44 expression by prostate adenocarcinomas predicts a poor prognosis, independent of stage and grade (10).

In the present study, we demonstrate that transgenic expression of standard rat CD44 isoform in highly metastatic AT3.1 rat prostate cancer cells results in the suppression of metastasis without suppressing tumorigenicity. However, using a non-hyaluronate-binding standard rat CD44 isoform mutant, we demonstrated that this metastasis suppression is independent of binding to hyaluronate. These results are consistent with a previous study using the Dunning R-3327 rat prostate cancer system that demonstrated that the metastatic ability of the various sublines in this system does not correlate with the ability of the sublines to bind to hyaluronate (16).

Although hyaluronate is the principle ligand of the standard CD44 isoform, the standard CD44 isoform can adhere to other extracellular matrix-associated proteins such as collagen (19), fibronectin (20), laminin (20), and chrondroitin sulfate (21). It is possible that hyaluronate-independent metastasis suppression by the standard CD44 isoform requires ligands other than hyaluronate. These ligands are currently being tested to identify which one is required for metastatic suppression by the standard CD44 isoform in the Dunning R-3327 rat prostate cancer system.

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