Expression of the CD44v2-10 Isoform Confers a Metastatic Phenotype: Importance of the Heparan Sulfate Attachment Site CD44v3

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ABSTRACT

We expressed the full-length CD44v2-10 isoform in SKHep1 cells, a nonmetastatic human hepatocellular carcinoma cell line that does not express any endogenous CD44v isoforms. In SCID mice, expression of CD44v2-10 by SKHep1 cells had no effect on s.c. primary tumor development but caused pulmonary metastases in 41% (7 of 17) of animals compared with control SKHep1 cells (0 of 16; P < 0.01). CD44v2-10 expression by SKHep1 cells resulted in enhanced heparan sulfate (HS) attachment and an enhanced capacity to bind heparin-binding growth factors. Mutation of the v3 domain to prevent HS attachment and growth factor binding abolished the metastatic phenotype, demonstrating that HS modification of CD44v2-10 plays a critical role in the development of metastases in this model. However, in vitro proliferation, motility, and invasion were not altered by CD44v2-10 expression.

INTRODUCTION

CD44 is a widely distributed transmembrane cell adhesion molecule that is the major cell surface receptor for HA (1). CD44 is implicated in a number of important cellular functions including lymphocyte homing to Peyers patches, leukocyte activation, lymphopoiesis, extracellular matrix adhesion, embryogenesis, and wound healing (reviewed in Ref. 2). Multiple high molecular weight isoforms of the core (CD44s) molecule may be generated through alternative splicing of up to 10 variable exons encoding part of the extracellular domain, referred to collectively as CD44v (3). CD44v2-10, together with its full cytoplasmic domain, represents the largest of the known human CD44 variant molecules. Up-regulation of expression of CD44s and CD44v characterizes many human malignancies, including colorectal metastases (Ref. 4 and reviewed in Ref. 5), breast, pancreatic, and gastric cancers, and in melanoma (reviewed in Ref. 2). Our previous studies have demonstrated that CD44v2-10 is expressed preferentially by human colorectal liver metastases compared with primary, nonmetastatic tumors (4). This variant isoform contains multiple exons with the potential to play functional roles in the metastatic process. Functional experimental studies have demonstrated a role for v6 (6) and v10 (7) in epithelial tumor metastasis in animal models. In antisense RNA studies, we have demonstrated that reduced expression of CD44v6 and CD44v3 in HT29 cells, whereas not affecting cell proliferation or adhesion to HA in vitro, abrogated the formation of liver and wound metastases after intrasplenic injection into nude mice (8). The variant exon v3 may be modified by HS that confers the capacity to bind heparin-binding growth factors in vitro (9). This may have the effect of increasing the local concentration of growth factors at the cell surface, promoting activation of high-affinity growth factor receptors (reviewed in Ref. 10). CD44v3 expression is up-regulated in a variety of human tumors and metastases (4, 11), and correlates with breast cancer progression. It has been suggested that CD44v3-HSPG expression may promote the metastatic phenotype through the sequestration and presentation of heparin-binding growth factors (12).

Here we have tested the functional role of the full-length CD44v2-10 isoform in tumor growth and metastasis. We developed a novel model of spontaneous metastasis using SKHep1 cells, a human hepatocellular cancer cell line that constitutively expresses only CD44s and not CD44 variant isoforms. The results show, for the first time, that the full-length CD44 splice variant can confer a metastatic phenotype on a human cancer and that the v3 HS attachment site is critical for this metastatic phenotype.

MATERIALS AND METHODS

Antibodies, Enzymes, and Growth Factors. The recombinant human growth factors bFGF (146 amino acid isoform), HB-EGF (148 amino acid isoform), HGF/SF (728 amino acid isoform), and VEGF (165 amino acid isoform) were obtained from R&D Systems (Abingdon, United Kingdom). The mouse antihuman CD44s antibody F10.44.2 (isotype IgG2a) was obtained from Serotec (Oxford, United Kingdom). The mouse antihuman CD44v3 (3G5, isotype IgG1), and CD44v6 (2F10, isotype IgG1) mAbs, antihuman HGF (24612.111, isotype IgG1), and antihuman VEGF (26503.111, isotype IgG1) neutralizing mAbs were obtained from R&D Systems. Fluorescein-conjugated SP, and the Biotin-SP-conjugated AffiniPure goat antimouse IgG (H+L) secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The antihuman CD44v9 mAb was purified from ascites derived from BALB/c mice injected i.p. with the murine hybridoma cell-line FW11.24.7 (European Collection of Cell Cultures No. 93070775). Heparitinase-I and antidesaturated uronate from heparitinase-treated HS ("AHS stub") 3G10 were obtained from Seikagaku America.

Cell Culture. SKHep1 (American Type Culture Collection; HTB52) is a human hepatocellular carcinoma cell line, and HTM29 is a metastatic derivative of HT29 cells (American Type Culture Collection; HTB38) that was obtained from Dr. Elise Kohn, NIH, Bethesda, MD. Cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamate, 25 mM HEPES, 60 mg/ml penicillin G, 100 mg/ml streptomycin sulfate, and 1 mM sodium pyruvate in a 5% CO2 and 95% air atmosphere at 37°C. Cell viability was determined by trypan blue dye exclusion.

DNA Constructs. A neomycin-resistant eukaryotic expression plasmid containing the human EF-1α promoter (pEFDNA3) was generated by inserting a 1.2-kb blunt-ended HindIII DNA fragment encoding the EF-1α promoter from pEFBOS-H3 (derived from Ref. 13) into the blunt-ended HindIII/NruI restriction enzyme site of pcDNA3 (Invitrogen), thereby replacing the existing

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4 The abbreviations used are: HA, hyaluronic acid; FACS, fluorescence-activated cell sorting; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; VEGF, vascular endothelial growth factor; mAb, monoclonal antibody; SP, streptavidin; EF-1α, elongation factor-1α; IF, immunofluorescence.
cytomegalovirus and T7 promoter regions. The 2270-bp cDNA encoding CD44v2-10 was isolated from a human HT29 cDNA library in pCDM8 and cloned into the EcoRV site of pEFDNA3. This construct was designated pCDNA3/CD44v2-10.

The CD44 signal peptide (encoded by exon 1) was replaced with the CD33 signal peptide, which has been shown previously to improve the efficiency of CD44v-Rg expression and is cleaved after secretion (14). To replace the CD44 signal sequence (exon 1) with the CD33 signal sequence, a 391-bp DNA fragment encoding the CD33 signal sequence was amplified by PCR from pCD44v3-10-lg (14). The forward amplification primer was CD33F: 5'−TAGTGAATCTGATATACTAAGTTCCATGCATCCTGTCATTAAAGC−3', which contains the restriction enzyme sites KpnI, EcoRI, and HindIII (italicized). The reverse amplification primer was CD44BamR: 5'−GGTGGATCCGCCGGAATCCACCATCAGTCCTC−3', containing a BamHI restriction enzyme site. PCR was performed using PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA), and the reaction was incubated at 94 °C for 1 min, 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, cycled 30 times. The amplified 1.1-kb DNA was cloned into the KpnI/BamHI site of pCD44v2-10cDNA3 generating pCD44v2-10/EFDNA3.

The CD44v2-10HSM was generated by in vitro site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer’s instructions. T→G mutations at nucleotides 862 and 868 were introduced, altering the Ser-Gly-Ser-Gly HS attachment motif in CD44v3 to Ala-Gly-Ala-Gly. The synthesized oligonucleotides used were 7'HSM: 5'−CTTGCATTGTGGATCGAGGATT/CTGAGG (mutant nucleotides are italicized).

PCR reactions were carried out following the manufacturer’s instructions, and the resultant mutant clones were verified by DNA sequence analysis.

**Generation of Stable Transfectants.** SKHep1 cells (2 × 10⁶) in log phase were combined with 20 μg of purified pCD44v2-10/EFDNA3, pCD44v2-10HSM/EFDNA3, or pEFDNA3 vector alone, and electroporated at 250 V and 960 μF. Clones resistant to 0.8 mg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) were isolated and analyzed for CD44s and CD44v expression by FACS analyses. Two independent lines demonstrating the Inc., Gaithersburg, MD) were isolated and analyzed for CD44s and CD44v2-10 was isolated from a human HT29 cDNA library in pCDM8 and cloned into the EcoRV site of pEFDNA3. This construct was designated pCDNA3/CD44v2-10.

One 10⁶ viable cells (SKHep1, SKHep1/NEO, SKHep1/CD44v2-10 clone #7, SKHep1/CD44v2-10 clone #13, SKHep1/CD44v2-10HSM clone #7, and SKHep1/CD44v2-10HSM clone #9) suspended in 0.05 ml of PBS were injected s.c. into the left flank of each mouse. All of the cell lines were tested in the same experiment. The mice were sacrificed after 10 weeks or sooner if moribund. Flask tumors were excised and measured in three dimensions with digital calipers. Representative samples from the liver and lung (inflated with 1 ml of Bouin’s Fixative), and the flank sites were preserved for histological examination by fixation in 10% buffered formalin followed by paraffin embedding. These studies were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes. The procedures were approved through the Bancroft Centre Research Ethics Committee (approval number A90302012).

**Immunohistochemistry.** Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections of tumor tissues by the avidin-biotin complex method, with the following exceptions. Sections of tumor tissues on glass slides were dried, deparaffinized, and stained using antihuman CD44s, CD44v3, CD44v6, or CD44v9 mAbs. Nonspecific antibody binding was inhibited using the Vector M.O.M. Immunodetection kit (Vector Laboratories, Burlingame, CA).

**RESULTS**

**Generation of CD44v2-10-expressing Tumor Cell Lines.** The human CD44v2-10 cDNA encodes all of the alternatively spliced exons in the proximal extracellular domain, and includes the transmembrane and cytoplasmic domains (there is no CD44v1-10 because human CD44v1 contains a stop codon). CD44v2-10 was isolated from a human HT29 cDNA library and inserted into the mammalian expression vector pEFDNA3 under the control of the human EF-1α promoter. The CD44 signal peptide in exon 1 was replaced with the CD33 signal peptide to increase efficiency of membrane expression (14). The resulting construct (pCD44v2-10/EFDNA3) is schematically represented in Fig. 1A.

To investigate the function of CD44v2-10 in metastasis, pCD44v2-10/EFDNA3 and the plasmid vector alone as a negative control (pEFDNA3/NEO) were each transfected into SKHep1 cells, and a
CD44v2-10 and CD44v9 (data not shown). Primary tumors formed by addition to the absence of CD44v3, these sections were negative for staining of the primary tumors formed by parental SKHep1 and SKHep1/NEO cell lines were strongly positive for expression of core domains.

In vivo, 889 mice were s.c. inoculated with SKHep1/NEO and parental SKHep1 cells. Primary tumor development was observed at the s.c. sites 2 weeks after injection, and all of the mice developed primary tumors during the 10-week course of the experiment. The median tumor volumes are recorded in mm³, with minimum and maximum tumor volumes for each clone displayed in parentheses.

Expression of CD44v2-10 by SKHep1 Cells Confers a Metastatic Phenotype in Vivo. To test the metastatic propensity of the CDv2–10 isoform in vivo, a mouse model of spontaneous metastasis was established. The human HCC cell line SKHep1 forms tumors after s.c. inoculation in SCID mice but does not form spontaneous lung metastases. Groups of 8–9 mice were s.c. inoculated with 1 × 10⁶ SKHep1 cells expressing CD44v2-10 (clones #7 and #13), SKHep1/NEO cells, or parental SKHep1 cells. Primary tumor development was observed at the s.c. sites 2–3 weeks after injection, and all of the mice developed primary tumors during the 10-week course of the experiment. The mice were sacrificed when the majority of the primary tumors in the control group were ~1 cm³.

There was no significant difference in primary tumor size between mice inoculated with the control SKHep1 cell lines and those expressing CD44v2-10 (Table 1). In contrast, histological examination of the lungs of the mice revealed the presence of pulmonary metastases of SKHep1 cells expressing CD44v2-10 (7 of 17), whereas the lungs of animals injected s.c. with SKHep1/NEO and parental SKHep1 cells were free of tumor (0 of 16; P < 0.01). These results demonstrate that expression of CD44v2-10 promotes the metastasis of SKHep1 cells in vivo.

Expression of CD44v2-10 Is Maintained in Vivo. Immunostaining of the primary tumors formed by parental SKHep1 and SKHep1/NEO cell lines were strongly positive for expression of core CD44 but were negative for CD44v3, as shown in Fig. 2, A–F. In addition to the absence of CD44v3, these sections were negative for CD44v6 and CD44v9 (data not shown). Primary tumors formed by SKHep1/CD44v2-10 cell lines (and SKHep1/CD44v2-10HSM cell lines, see below) displayed strong membrane staining for core CD44 and CD44v3 (Fig. 2, G–L), as well as for CD44v6 and CD44v9 (data not shown). Pulmonary metastases from mice inoculated with SKHep1/CD44v2-10 maintained strong membrane staining for core CD44 and CD44v3 protein expression (Fig. 2, M–O), as well as for CD44v6 and CD44v9 (data not shown).

SKHep1 Cells Expressing CD44v2-10 Exhibit HS Attachment to CD44v3 in Vitro. To determine whether CD44v2-10 expression by SKHep1 cells results in HS attachment to CD44v3, SKHep1 parental, SKHep1/NEO, SKHep1/CD44v2-10, and SKHep1/CD44HSM cells were heparitinase- or PBS-treated, and cell lysates were subjected to Western blot analysis using an antibody directed to HS stubs. Blots were stripped and reprobed with an anti-CD44v3 mAb. A band of M, 250,000 corresponding to CD44v2-10 was detected in SKHep1/CD44v2-10 cells (Fig. 3) and was not seen in control cell lines. This band was not seen in SKHep1/CD44HSM cell lysates. These data show that CD44v2-10 is modified by HS at the CD44v3-HS attachment site, when expressed in SKHep1 cells.

**Table 1. Expression of CD44v2-10 confers a metastatic phenotype on SKHep1 cells in vivo.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Primary tumor volumeᵃ⁻ᵇ</th>
<th>Lung metastasesᵍ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKHep1 wt</td>
<td>602 mm³ (120; 936)</td>
<td>0/8</td>
</tr>
<tr>
<td>SKHep1 NEO</td>
<td>448 mm³ (84; 1037)</td>
<td>3/9</td>
</tr>
<tr>
<td>SKHep1/CD44v2-10 #7</td>
<td>352 mm³ (99; 576)</td>
<td>3/9</td>
</tr>
<tr>
<td>SKHep1/CD44v2-10 #13</td>
<td>724 mm³ (240; 1056)</td>
<td>0/8</td>
</tr>
<tr>
<td>SKHep1/CD44v2-10HSM #7</td>
<td>300 mm³ (160; 693)</td>
<td>0/8</td>
</tr>
<tr>
<td>SKHep1/CD44v2-10HSM #9</td>
<td>444 mm³ (125; 910)</td>
<td>0/8</td>
</tr>
</tbody>
</table>

ᵃ The median tumor volumes are recorded in mm³, with minimum and maximum tumor volumes for each clone displayed in parentheses.

ᵇ The differences between the incidence of lung metastases in the combined control groups compared with the incidence of lung metastases in mice injected with each of the CD44v2-10 transfectants are: SKHep1 wt + NEO versus CD44v2-10 #7, P = 0.0365; SKHep1 wt + NEO versus CD44v2-10 #13, P = 0.0066, Fisher’s exact test.

Fig. 2. Immunostaining of tumors formed in SCID mice inoculated s.c. with control and CD44v2-10-expressing SKHep1 cell lines. Photomicrographs of paraffin sections from primary tumors derived from parental SKHep1 cells (A–C), SKHep1/NEO (D–F), CD44v2-10 (G–I), or CD44v2-10HSM (J–L). Photomicrographs of paraffin sections from pulmonary metastases derived from SKHep1/CD44v2-10 cells (M–O). Sections B, E, H, K, and N were stained with anti-CD44 core antibody (F10.44.2), and sections C, F, I, L, and O were stained with anti-CD44v3 antibody (3G5). Sections A, D, G, J, and M are negative controls stained with secondary antibody only.

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CD44v2-10 CONFERS A METASTATIC PHENOTYPE

Expression of CD44v2-10 Decreases SKHep1 Cell Adhesion to Hyaluronan. The effect of CD44v2-10 expression by SKHep1 cells on hyaluronan binding was examined by adhesion assays on immobilized hyaluronan. Parental SKHep1 and SKHep1/NEO cell lines

CD44v2-10-expressing SKHep1 Cells Bind Heparin-binding Growth Factors in Vitro: Requirement for CD44v3. To investigate the binding of heparin-binding growth factors to SKHep1/CD44v2-10 cells, quantitative ligand-binding analyses were performed. HGF/SF and VEGF binding to the cells was measured by FACS using anti-HGF/SF and anti-VEGF mAbs. bFGF binding was determined by measuring the adhesion of the cells to bFGF-coated PVC plates. Parental SKHep1 and SKHep1/NEO control cell lines displayed low levels of binding with bFGF, HGF/SF, and VEGF, and bFGF heparin-binding growth factors, respectively (Fig. 3, B–D). SKHep1/CD44v2-10HSM cells, in which the HS attachment site was mutated, displayed heparin-binding growth factor binding similar to that of the control cell-lines (Fig. 3, B–D). SKHep1/CD44v2-10 growth factor binding was competitively inhibited by excess heparin or treatment with heparitinase (data not shown). These data demonstrate that growth factor binding is dependent on a functional CD44v3 isoform and additionally show that HS modification of CD44v3 is required for growth factor binding.

CD44v2-10 Expression Does Not Change in Vitro Tumor Cell Proliferation, Cell Migration, Invasion, or Motility. To investigate whether expression of CD44v2-10 by SKHep1 cells may initiate an autocrine loop to enhance growth factor binding, and hence, the proliferation of SKHep1 cells in vitro, cell growth was monitored over a period of 3 days in the absence and presence of added HB-EGF (10 pg to 10 nm), bFGF (10 pg to 10 nm), and HGF/SF (10 pg to 10 nm). No differences were found between the proliferation rates of SKHep1/CD44v2-10, SKHep1/CD44v2-10HSM, SKHep1/NEO, or parental SKHep1 cells in the absence or presence of added growth factors (data not shown). These results suggest that CD44v2-10-mediated metastasis is not dependent on increased proliferation of SKHep1 cells. Increased cell migration and invasion are key phenotypic advantages of malignant cells favoring metastasis. To determine whether expression of CD44v2-10 by SKHep1 cells promotes increased cell migration, SKHep1 parental, SKHep1/NEO controls, SKHep1/CD44v2-10, and SKHep1/CD44v2-10HSM cells were subjected to in vitro monolayer wound ing in wells (on coverslips) with and without HA coating. No difference in cell migration was seen between any of the cell lines. The same studies were repeated in the presence of exogenous growth factors bFGF and HGF/SF, with no differences observed. Cell invasion was studied in Matrigel-coated transwells. In separate studies, BSA and BSA with bFGF were used as attractants. Additional studies used cells pretreated with PMA in serum-free medium. In none of the studies did cells migrate or invade Matrigel. Transwell migration studies were performed with and without HGF as an attractant, and in the presence and absence of HA and Matrigel. None of the cell lines migrated through the Matrigel, and no differences in cell migration were observed between any of the clones. These studies did not demonstrate in vitro phenotypic differences between parental SKHep1 cells and NEO controls, and SKHep1/CD44v2-10 and SKHep1/CD44v2-10HSM cells in migration or invasion.

HS-modified CD44v3 Is Necessary for the Metastasis of SKHep1 Cells. To investigate the role of HS modification of the CD44v3 domain in CD44v2-10-mediated experimental metastasis, a mutant construct was designed to alter the Ser-Gly-Ser-Gly HSattachment site to Ala-Gly-Ala-Gly (shown schematically in Fig. 1A). An identical mutation has been shown by others to eliminate the attachment of HSv to CD44v3 isoforms, preventing the binding of bFGF to fusion proteins containing CD44v3 (16). The mutant construct (pCD44v2-10HSM/EFDNA3) was transfected into SKHep1 cells, and stable cell lines were selected. Two cell lines with high cell surface CD44v2-10 expression were selected by FACS. Both cell lines showed substantial expression of core CD44v3, v3, v6, and v9 at levels similar to SKHep1/CD44v2-10 (data not shown). Analysis of CD44v3 expression in each of the parental SKHep1, SKHep1/NEO, SKHep1/CD44v2-10, and SKHep1/v2-10HSM cell lines by Western blot analysis using an antibody specifically directed to the core CD4 domain showed similar levels of the Mr 90,000 CD44s isoform (data not shown).

Two SKHep1/CD44v2-10HSM-expressing cell lines were inoculated s.c. into SCID mice. Primary tumors developed within 10 weeks at a similar rate to that observed for SKHep1/CD44v2-10 cells, SKHep1/NEO cells, and parental SKHep1 cells. There was no significant difference in primary tumor size between the CD44v2-10HSM and the other cell-lines (Table 1). In contrast, histological examination of the lungs revealed the absence of pulmonary metastases in mice inoculated with SKHep1 cells expressing CD44v2-10HSM (0 of 16), compared with mice bearing CD44v2-10-expressing tumors (7 of 17; P < 0.01). These results demonstrate that HS modification of v3 is required for metastasis of CD44v2-10-expressing SKHep1 cells in vivo.

Protein Expression and Primary Tumor Growth. Higher levels of CD44v2-10 expression were selected by FACS. Both SKHep1/CD44v2-10 cells showed increased cell adhesion to bFGF. Cells were allowed to adhere to bFGF precoated plates (0.313 ng/ml), and incubated for 1 h at 37°C before washing. Growth factor binding was detected by FACS using antihuman HGF and antihuman VEGF antibodies, respectively. D. cell adhesion to bFGF. Cells were allowed to adhere to bFGF precoated plates (0.313 µg/ml), and incubated for 1 h at 37°C before washing and detection by crystal violet assay. bFGF binding was completely abolished by pretreatment with heparin (200 µg/ml) or treatment with heparitinase (data not shown). CD44v2-10-expressing SKHep1 cells were subjected to heparitinase. CD44v2-10 and CD44v2-10HSM are detected using anti-v3 mAb 3G5 (R&D Systems), and are seen at Mr 280,000. Faint, nonspecific bands can be seen at Mr 300,000 using the mAb 3G10 and do not represent CD44 because they are not detected by the anti-CD44v3 mAb 3G5. B-D. binding of heparin-binding growth factors and hyaluronan to control and CD44v2-10-expressing SKHep1 cells. Each cell line was incubated with (B) 10 ng HGF/SF or (C) 40 ng VEGF for 1 h at 4°C before washing. Growth factor binding was detected by FACS using antihuman HGF and antihuman VEGF antibodies, respectively. D. cell adhesion to bFGF. Cells were allowed to adhere to bFGF precoated plates (0.313 µg/ml), and incubated for 1 h at 37°C before washing and detection by crystal violet assay. bFGF binding was completely abolished by pretreatment with heparin (200 µg/ml) for 15 min at room temperature. E. binding of CD44v2-10-expressing and control SKHep1 cells to immobilized hyaluronan. Each cell line indicated was allowed to adhere to hyaluronan precoated plates (5 mg/ml) for 16 h at 4°C in the presence of PBS, 250 µg/ml potassium hyaluronan, or 500 µg/ml potassium hyaluronan. Results are the average of three separate experiments; bars, ±SE. * soluble HA at 500 µg/ml abolishes binding of all cell lines to immobilized HA.
displayed high levels of binding to immobilized hyaluronan, which was effectively competed out by preincubation with soluble hyaluronan (Fig. 3E). In contrast, SKHep1/CD44v2-10 cells showed an 
~3-fold reduced affinity for hyaluronan. SKHep1/CD44v2-10HSM cells demonstrated a level of binding similar to that of the control cell lines. These data show that HS modification of CD44v3 interferes with CD44-hyaluronan interactions, a result in agreement with Bennett et al. (17). Thus, CD44v2-10 expression by SKHep1 cells has a dominant-negative effect on hyaluronan binding, leading to a less adhesive phenotype.

DISCUSSION

This study has shown for the first time that expression of the full-length CD44v2-10 variant isoform can confer a metastatic phenotype on a human epithelial cancer. Whereas a metastatic phenotype has been conferred in an animal model using CD44v isoform overexpression in a rat-derived cancer cell line (6), this has not been demonstrated previously in an in vivo human cancer cell model. The finding in previous studies that various variant exon combinations have induced phenotypes with metastasis-associated properties suggests that multiple domains in the CD44 molecule may be involved in tumor metastasis. Our approach was to determine whether the full-length CD44v2-10 variant could induce the metastatic phenotype in human cancer cells, and if so, to dissect the possible contributions of specific exon domains. On the basis of our previous finding that it was the CD44v2-10 variant that was identified uniquely in metastases of colorectal tumors (4), we expressed this full-length variant CD44 isoform in SKHep1 cells, a cell line that is nonmetastatic after s.c. inoculation in nude SCID mice, which conferred a metastatic phenotype, demonstrating that CD44v2-10 very likely contributes functionally to metastatic behavior.

Although CD44 variant isoforms have been implicated in the metastatic process in a number of clinical and experimental studies, the precise mechanisms by which tumor cells exploit CD44 are not well understood. CD44v3 is a domain that can be modified by HS attachment (16). We found that an intact HS attachment site in CD44v3 was essential for CD44v2-10-mediated metastasis in the SKHep1 experimental tumor model. Moreover, SKHep1 cells expressing HS-modified CD44v2-10 displayed an enhanced ability to bind the heparin-binding growth factors HGF/SF, bFGF, and VEGF compared with cells expressing a HS-deficient mutant CD44v2-10 (CD44v2-10HSM) and SKHep1 control cell lines. This finding is consistent with those of other authors (9, 12) who have shown that the HS-modified CD44v3 HS binding domain binds heparin-binding growth factors in vitro. It is believed that HSPGs, such as CD44v3-HSPG, act as high-capacity, low-affinity receptors that increase the concentration of heparin-binding growth factors at the cell surface, thereby potentially enhancing high-affinity growth factor receptor binding and activation. The effect of HSPGs is most apparent at submaximal concentrations of growth factor, enhancing the effects of the locally available growth factors (18). Because CD44v3-HSPG can promote c-Met activation (12), we addressed the possibility that SKHep1 cells expressing CD44v2-10 are able to sequester and present heparin-binding growth factors in an autocrine loop, promoting tumor cell activation in vivo proliferation and motility. However, CD44v2-10-expressing cells did not display increased cell proliferation in vitro, even after incubation with exogenous HGF/SF, bFGF, HB-EGF, or VEGF compared with control cell lines. Furthermore, primary tumors derived from s.c. inoculation of SKHep1/CD44v2-10 cells in the mouse did not grow more rapidly and were not larger than those derived from control cell lines. These results suggest that the mechanism of CD44v2-10-mediated metastasis in SKHep1 cells is unlikely to be that of enhanced (autocrine) cell proliferation.

Other properties considered important in the metastatic cascade include increased cellular motility and invasion, which can be mediated by certain heparin-binding growth factors including HGF/SF. SKHep1/CD44v2-10 cells did not demonstrate enhanced migration or invasion in vitro, even after incubation with exogenous HGF/SF. Moreover, immunoprecipitated h-met from SKHep1/CD44v2-10 cells did not demonstrate enhanced tyrosine phosphorylation of h-met with HGF stimulation (data not shown). These experiments are at variance with previous findings in Namalwa cells double-transfected with CD44v3-10 and c-met under HGF stimulation, where enhanced tyrosine phosphorylation of c-met was demonstrated (12). However, this Namalwa experimental system is not spontaneously metastatic in vivo and, hence, may not be directly relevant to tumor metastasis.

There are other possible mechanisms for CD44v2-10-mediated metastasis in SKHep1 cells. Cell surface CD44v3-HSPG cannot only efficiently stimulate bFGF-mediated proliferation in a cell-line transfected with fibroblast growth factor receptor-1, but macrophage CD44v3-HSPG can present bFGF to adjacent cells in inflamed synovial tissue (19). Such findings suggest that SKHep1 cells expressing CD44v3-HSPG may use similar paracrine regulation of growth factor function. This would allow tumor cells to modify their microenvironment, such as establishing chemotactic gradients for endothelial cells and stromal fibroblasts. Such a two-way paracrine exchange of growth factors between tumor cells and vascular endothelial cells has been demonstrated clearly by Fukumura et al. (20). The potential relationship between CD44v3-HSPG-expressing tumor cells, microvascular endothelial cells, and host stromal cells warrants additional study.

An alternative mechanism for the observed metastatic behavior might be through signal transduction. There is accumulating evidence that, in general, adhesion molecules participate not only in mediating cell adhesion, but also in signal transduction (21, 22). CD44 has been implicated in signal transduction involving Src kinases, including Lck, Fyn, Lyn, and Hck in lipid rafts (23), and the Rho family of proteins through association with ERM proteins. It is conceivable that on growth factor binding by CD44v3, outside-in signaling may influence pathways associated with metastatic behavior.

Several studies have shown that metastatic behavior is dependent on CD44-HA interactions (24), whereas others have demonstrated that CD44 variant isoforms confer a metastatic phenotype independent of HA binding (25). The data presented here demonstrate that CD44v2-10 expression in SKHep1 cells conferred reduced HA binding and a metastatic phenotype, which may indicate that reduced HA binding is important for metastasis in this experimental model. HS-modified CD44v3-containing isoforms have been shown to have a reduced affinity for HA that is restored by removal of HS side chains (19). Consistent with these findings, CD44v2-10HSM expression in SKHep1 cells did not alter HA binding nor promote metastasis compared with control SKHep1 cell lines in this model, suggesting that either HA binding is not important or that metastasis requires both reduced HA binding and an intact CD44v3 domain. Additional experiments will be required to clarify the role of HA binding in metastasis in this model.

This is the first study demonstrating that HS-modified CD44v2-10 confers a metastatic phenotype on human cancer cells in vivo but that the mechanism is unlikely to be through an autocrine enhancement of tumor cell proliferation or migration. These findings suggest that strategies directed against CD44v3-HA attachment could provide realistic antimetastasis therapies.
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Expression of the CD44v2-10 Isoform Confers a Metastatic Phenotype: Importance of the Heparan Sulfate Attachment Site CD44v3

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