Interaction between CD44 and Hyaluronate Is Directly Implicated in the Regulation of Tumor Development

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Summary

CD44 is implicated in the regulation of tumor growth and metastasis but the mechanism by which expression of different CD44 isoforms determines the rate of primary and secondary tumor growth remains unclear. In the present study we use a human melanoma transfected with wild-type and mutant forms of CD44 to determine which functional property of the CD44 molecule is critical in influencing tumor behavior. We show that expression of a wild-type CD44 isoform that binds hyaluronic acid augments the rapidity of tumor formation by melanoma cells in vivo, whereas expression of a CD44 mutant, which does not mediate cell attachment to hyaluronate, fails to do so. The importance of CD44-hyaluronate interaction in tumor development is underscored by the differential inhibitory effect of soluble wild-type and mutant CD44-Ig fusion proteins on melanoma growth in vivo. Whereas local administration of a mutant, nonhyaluronate binding, CD44-Ig fusion protein has no effect on subcutaneous melanoma growth in mice, infusion of wild-type CD44-Ig is shown to block tumor development. Taken together, these observations suggest that the tumor growth promoting property of CD44 is largely dependent on its ability to mediate cell attachment to hyaluronate.

CD44 is a broadly distributed cell surface glycoprotein implicated in multiple physiologic cellular functions including cell–cell adhesion (1–3), lymphocyte activation (3–6), and cell–substrate interaction (7–11). Characterization of the genomic clone of CD44 has demonstrated the existence of 10 exons encoding a portion of the extracellular domain that are differentially spliced (12), giving rise to a number of isoforms with different molecular masses (9, 13–17). In addition to variable exon usage, cell type–specific glycosylation may contribute to structural and functional CD44 isoform diversity (14, 18).

Expression of the different isoforms is not uniform. The most widely expressed isoform is the 80–90-kD glycoprotein that represents the "standard" CD44 molecule, as it does not contain differentially spliced exons (13, 19). It is commonly referred to as CD44H and has been shown to be a major cell surface receptor for hyaluronate (8, 10, 20). CD44H is the principal isoform found in hematopoietic cells, fibroblasts, melanomas, and some epithelial cells (9, 13, 17, 21). Larger isoforms that contain different combinations of differentially spliced exons have a more restricted pattern of expression and appear to be preferentially associated with epithelial cell subpopulations (9, 14, 15, 17, 22). Although all isoforms contain the hyaluronate (HA)[1]-binding domain, their affinity for surface-bound HA is variable (9, 23), and at least some of the larger isoforms appear unable to mediate attachment of cells to HA-coated surfaces (9, 11, 23).

CD44 expression has been associated with tumor growth and metastasis (16, 23) but the molecular basis for this association has remained unclear. In the rat, expression of a 160-kD variant form of CD44, containing variable exon 6 which corresponds to exon 10 of the genomic clone (12), has been proposed to induce metastatic behavior in a pancreatic carcinoma cell line (16). In experiments designed to directly test the effect of CD44 expression on lymphoma growth and dissemination, it was observed that expression of CD44H significantly enhanced local tumor formation in nude mice, whereas expression of CD44E, a 150-kD isoform associated with some carcinomas (9) and containing variable exons 8–10 (12–14 of the genomic clone) (12), failed to promote tumor growth (23). The difference in the rapidity of

A. Bartolazzi and R. Peach contributed equally to this paper.

[1] *Abbreviations used in this paper:* ECM, extracellular matrix; HA, hyaluronate; VLA, very late activation antigen.

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local and metastatic tumor formation between CD44H and CD44E-expressing lymphoma cells correlates with the difference in their capacity to adhere to surface-bound HA (9, 11, 23). It was proposed that host tissue HA might enhance tumor development by providing a molecular bridge which facilitates HA-receptor bearing tumor cell attachment to extracellular matrix (ECM) components. The ECM could serve as a scaffold for tumor growth as well as a reservoir of growth factors potentially beneficial to the malignant cells (24). In the present work we provide direct evidence that the capacity of CD44 to mediate tumor cell attachment to hyaluronate determines the rate of formation of the resulting tumor mass. We also show that soluble CD44 can be effectively used to contain local tumor growth in vivo.

Materials and Methods

Cell Lines. Development of stable CD44 transfectants in the human melanoma cell line MC has been described previously (11). MC44H, MC44E, MC44TL, and MC-T express wild-type CD44H, wild-type CD44E, a cytoplasmic tail deletion mutant of CD44H, and pSV2neo, the selection plasmid containing the neomycin resistance gene, respectively. Additional transfectants used in this study include MCα2,6ST, expressing the β-galactoside α-2,6-sialyltransferase; MC44H41R-A, expressing the mutant CD44H which does not bind HA (25); and MC44H/E expressing both CD44H and CD44E. These transfectants were generated by introducing each respective cDNA clone in a πH3M vector (26) modified to include a hygromycin resistance gene. Transfections were performed by electroporation (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) using the same conditions as those for the previous transfectants (250 V, 960 μF). Clones were selected for hygromycin resistance in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM glutamine (GIBCO BRL, Gaithersburg, MD), 10% FCS (Irvine Scientific), gentamycin, and 500 μg/ml Hygromycin B (Boehringer Mannheim Corp., Indianapolis, IN). To generate MC44H/E double transfectants, the CD44H construct in the vector containing the hygromycin resistance gene was introduced into MC44E transfectants containing the neomycin resistance gene (11). Clones were selected for resistance to hygromycin B and G418 (GIBCO BRL). All transfectants were periodically tested for expression of CD44 isoforms by indirect immunofluorescence.

The murine B16F10 melanoma was obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FCS and gentamycin.

mAbs and Polyclonal Antisera. The anti–human CD44 mAb F10-44-2, which recognizes all CD44 isoforms, was acquired from R & D Systems Inc. (Minneapolis, MN). Antimurine CD44 IM7 hybridoma was obtained from the American Type Culture Collection. The murine mAb M-kid 2, which recognizes a conformational epitope of the $\alpha3\beta1$ (very late activation antigen [VLA] 3) integrin has been previously described (27). mAbs to VLA1 and VLA4 integrins were kindly provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA); mAbs to VLA2 and VLA5 were a gift from Dr. Randy Byers (Massachusetts General Hospital). The rat mAb to VLA6 was purchased from Janssen Biochimica (Accurate Chemical & Scientific Corp., Westbury, NY).

Immunofluorescence. Cells were detached from plates with EDTA, washed, resuspended in PBS, and incubated with mAbs or polyclonal antisera (30–50 μg/ml) for 45 min at 4°C. Cells were washed in PBS, incubated with fluorescein-labeled $F(ab)_2$ fractions of goat anti–rat or goat anti–mouse affinity purified antibody (Cappel Laboratories, Malvern, PA) for 30 min at 4°C, washed, resuspended in PBS, and analyzed on a FACS® (Becton Dickinson & Co., Mountain View, CA).

For tissue staining, 5 μm frozen tissue sections were mounted onto slides, air-dried, and incubated with 10 μg/ml of each receptorglobulin or human IgG for 45 min at room temperature. The slides were washed in PBS, incubated with fluorescein-labeled goat anti–human affinity-purified antibody, washed, and examined under an epifluorescence microscope (Nikon Inc., Melville, NY).

Adhesion Assays. 96-well tissue culture plates were coated with 5 mg/ml hyaluronate, chondroitin sulfate, fibronectin, collagen type I or collagen type VI, or heat denatured BSA overnight at 4°C, and nonspecific sites were blocked with 1 mg/ml BSA. The wells were washed with PBS and seeded with ^{51}Cr-radiolabeled B16F10 melanoma or MC transfectant cells (10^5 cells/well) in PBS. Adhesion was allowed to proceed at 4°C for 30 min. The wells were then washed three times with PBS, the adherent cells were lysed with 1% SDS, and the lysate radiolabel was determined in a β-counter. In addition, 5 μ thick frozen tissue sections of mouse and rat skin, muscle, and kidney were prepared, mounted onto slides, air dried, and overlayed with MC transfectants (10^5 cells/slide) resuspended in PBS. Tissue section adhesion assays were performed at 4°C for 30 min with gentle rotational shaking. Slides were then washed in PBS, stained with toluidine blue (Sigma Chemical Co., St. Louis, MO) for 1 min, washed, and examined under an epifluorescence microscope. Hyaluronidase treatment of tissues was performed by incubating frozen tissue sections with 1 μg/ml streptomycete hyaluronidase (Calbiochem-Novabiochem, La Jolla, CA) in phosphate-buffered saline for 1 h at 37°C. Keratanase (0.5 U/ml) and heparitinase (0.01 U/ml) control digestions were performed under the same conditions.

Preparation of CD44 receptorglobulins and full-length CD44H mutant. CD44HRg (8), CD44HMutRg (24), and CD44ERg were prepared as previously described. Briefly, synthetic oligonucleotide-primed amplification of cDNA sequences encoding the extracellular sequences of CD44H and CD44H41R-A was performed by PCR. Oligonucleotide primers were designed to contain endonuclease restriction sites to facilitate subsequent insertion of amplified sequences in Ig expression vectors. Amplified CD44 sequences were subjected to appropriate restriction endonuclease digestion and ligated to Ig vectors previously subjected to corresponding endonuclease cleavage. CD44-Ig constructs were introduced into COS cells by the DEAE-Dextran method (8), and 5–8 d post transfection supernatants were harvested and receptorglobulins purified on protein A sepharose beads (Repligen, Cambridge, MA). Receptorglobulins were eluted with 0.1 M citric acid, pH 3.0, dialyzed overnight and purified protein concentration determined in ELISA assays (Amersham Corp., Arlington Heights, IL). To synthesize a full-length CD44H41R-A cDNA (including the transmembrane and intracytoplasmic domain-encoding segments), the Ig construct containing the mutant was digested with HindIII and AlwnI. The resulting fragment was mixed with an AlwnI/NotI-digested fragment derived from a vector containing full-length CD44H, and ligated together in a three-way ligation with a HindIII/NotI-digested CDM8 vector modified to contain a hygromycin resistance gene.

Determination of Cellular Growth Rate In Vitro. Each transfectant was cultured in 96-well flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA) at a concentration of 5 × 10^4 cells/well in DMEM/10% FBS. After 24 h of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine, cultured for an additional 24 h, harvested with an automatic pH.D harvester (Cambridge Bioscience, Cambridge, MA) and [^{3}H]thymidine incorporation determined in a β-counter. All cultures were done in triplicate.

Evaluation of Tumor Growth In Vivo. Pathogen-free 4–5-wk-old

SCID and 6–7-wk-old nude (*nu/nu*) mice were used for the study of in vivo tumor growth. MC melanoma transfectants were detached from tissue culture plates with EDTA, washed in sterile PBS and injected subcutaneously into SCID mice at a concentration of 6×10^6 cells/0.2 ml. Animals were examined three times a week for signs of calibrable tumor growth and were killed after 36–40 d and an autopsy performed. Tumor weight before autopsy was calculated by the formula $(d^2 \times D)/2$, where d represents the smallest and D the largest diameter of the tumor. To determine the role of CD44 in the promotion of B16F10 melanoma growth, nude mice were used (described below).

Blocking of Tumor Growth with Soluble CD44. Each animal was anesthesized with 2,2-dichloro-1,1-difluoroethyl-methyl ether (methoxyflurane) (Pitman-Moore, Inc., Mundelein, IL) in a semiclosed system and a single ALZET osmotic pump 1007D (Alza Corp., Palo Alto, CA), filled with 600 μg/0.1 ml of CD44HRg, CD44H41ARg, or human IgG, was implanted subcutaneously in the retroscapular region. Six mice/group were used. ALZET osmotic pumps 1007D are designed to deliver a fixed volume of 0.5 μl/h for a period of 7 d. The amount of receptorglobulin or IgG delivered is expressed by the formula $k = Q \times Cd$, where k is the mass delivery rate, Cd the concentration of the molecule used to fill the pump and Q the pumping rate (0.5 μg/h for the 1007D model). In this system, 3 μg/h of each receptorglobulin or IgG were delivered for 7 d. On the day after the implantation of the pump, B16F10 melanoma cells were detached with EDTA, washed in PBS, resuspended in CD44HRg, CD44Mut Rg, or human IgG at 125×10^3 cells/0.2 ml, and injected into the pocket containing the pump. Mice were killed at 8 and 14 d after the injection and tumor growth was assessed.

Radiolabeling and Immunoprecipitation. MC melanoma transfectants were washed and cultured in methionine-free medium (GIBCO BRL) supplemented with 10% dialyzed FCS for 2 h. Cells were then pulsed with 250 μCi/ml of [^{35}S]methionine (Amersham Corp.) for 12 h, washed in PBS, detached, and lysed in a buffer containing 1% Triton X-100, 10 μg/ml leupeptin, 100 U/ml aprotinin (all from Sigma Chemical Co.) and 10 μM PMSF (Bethesda Research Laboratories Inc., Bethesda, MD). After 1 h of lysis at 4°C, nuclei were removed by centrifugation and lysates were precleared with protein A–Sepharose CL4B (Pharmacia LKB, Uppsala, Sweden) coated with affinity purified rabbit anti–mouse IgG (Sigma Chemical Co.). After preclearing, lysates were incubated with protein A–Sepharose CL4B coated with anti-CD44 mAb F10-44-2 for 1 h at 4°C, the protein A–Sepharose beads washed, and the precipitates eluted by boiling. Immunoprecipitates were analyzed by SDS/7.5% PAGE. The gels were fixed, dried, and subjected to autoradiography.

To determine CD44 shedding, equal numbers of MC-T, MC44H, MC44E, and MC44H/E transfectants were radiolabeled with [^{35}S]methionine as above. 72 h after radiolabeling, culture supernatants were harvested, precleared, and incubated with anti-CD44 mAb F10-44-2 and protein A–Sepharose beads (Pharmacia LKB) at 4°C overnight. The beads were washed and precipitates eluted and analyzed by SDS-PAGE and autoradiography as above.

Results

A Soluble Form of a CD44H Mutant Does Not Bind Ligands In Vivo. Development of soluble CD44-Ig fusion proteins (termed CD44Rg, for receptorglobulins) has been instrumental in determining that CD44 is a receptor for hyaluronate (8). Recently, CD44HRg has been subjected to site-specific mutagenesis (25) and a single point mutation which modifies residue 41 from an arginine to an alanine has been observed to abrogate CD44-HA interaction (25). To determine whether CD44H41R-ARg (CD44MutRg) might recognize other ligands in the ECM, normal rat and mouse tissues were tested for reactivity with CD44MutRg by indirect immunofluorescence. CD44HRg bound to HA-containing areas in all organs, as previously demonstrated (8). However, CD44MutRg failed to bind the ECM of dermis, intestinal tissue (data not shown), skeletal muscle, and renal papilla (Fig. 1), indicating that the soluble CD44MutRg does not recognize ECM-bound HA and that potential interactions with other putative ligands are too weak to be revealed using this approach. These results provided a basis for comparing the mutant and wild-type CD44H in their capacity to enhance tumor growth in vivo and thereby assess whether CD44-HA interaction is the single most important factor in CD44-regulated tumor growth. A full-length CD44H41R-A cDNA, suitable for cell surface expression, was synthesized and expressed in COS cells. Anti-CD44 mAbs were found to react with the COS cell-expressed mutant CD44 but binding of fluoresceinated hyaluronate was abrogated (data not shown).

Expression of CD44 Polypeptides in a Human Melanoma Cell Line. A human melanoma cell line, MC (11), was stably transfected with CD44H41R-A in a hygromycin-resistance gene-containing expression vector and hygromycin-resistant clones selected and tested for surface CD44 expression. CD44H41R–A-expressing cells did not display an increase in their capacity to attach to HA-coated surfaces with respect to parental MC cells (data not shown). The same melanoma cell line had been used previously for stable expression of CD44H, CD44E, and CD44H cytoplasmic deletion mutants (CD44TL) in experiments designed to assess the role of CD44 in tumor cell adhesion to hyaluronate as well as migration on substrates in vitro (11). These studies had established that CD44H transfectants bind strongly to HA-coated surfaces whereas CD44E transfectants behave similarly to MC cells transfected with the selection plasmid only. MC cells transfected with a CD44H cytoplasmic domain deletion mutant attached to HA-coated surfaces weakly (11), but displayed no increase in migration (11). An additional cell line was developed, by transfecting the MC44E cell line with a CD44H cDNA in a hygromycin-resistance vector. The resulting clones were selected for resistance to both hygromycin and neomycin. CD44H/E double transfectants provided a means to assess whether coexpression of different isoforms with different HA binding potentials might have a distinct effect on tumor development.

All transfectants were compared for CD44 expression by indirect immunofluorescence using the F10-44-2 anti-CD44 antibody that recognizes all isoforms (Fig. 2). In addition, immunoprecipitation of lysates from [^{35}S]methionine radiolabeled transfectants was performed, partly because the relative expression of CD44H and CD44E in double transfectants could not be distinguished on the basis of immunofluorescence alone (Fig. 3). For in vivo experiments, transfectants were selected for comparable levels of cell surface CD44 expression. MC44H41R-A and MC44H cells displayed similar reactivity with anti-CD44 mAb as assessed by indirect im-

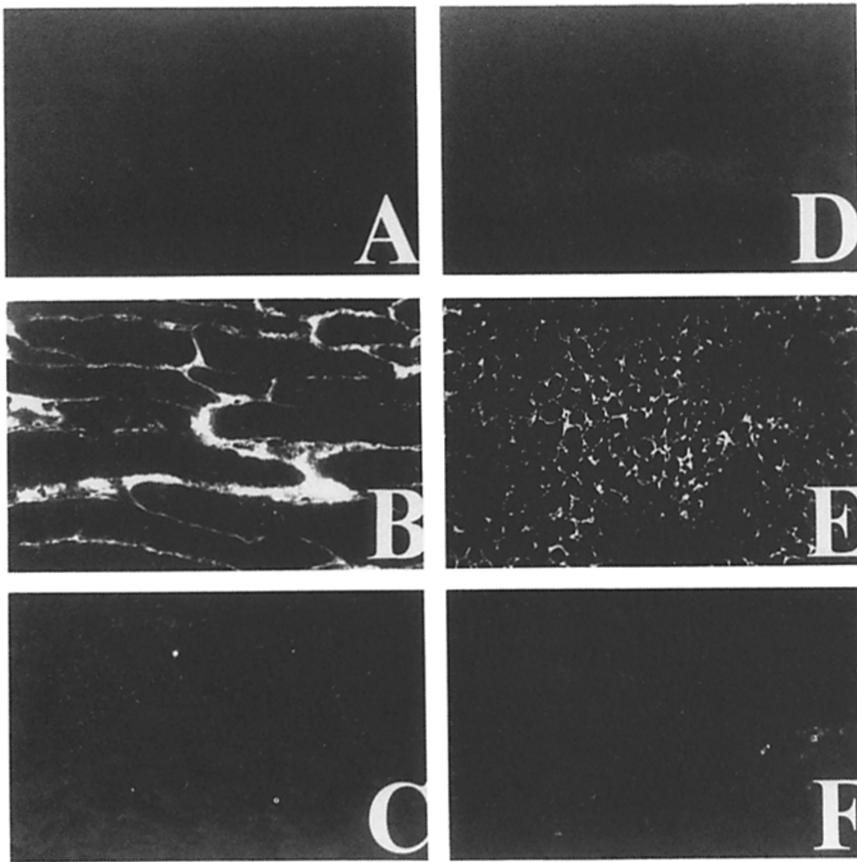


Figure 1. Binding of CD44HRg and CD44MutRg to ECM in vivo. Murine muscle and renal tissue were snap-frozen and 5-μm sections were cut, mounted onto slides, and stained. Muscle (*A–C*) and renal papilla (*D–F*) reactivity are shown with CD44 MutRg (*A* and *D*), CD44HRg (*B* and *E*), and human IgG (*C* and *F*).

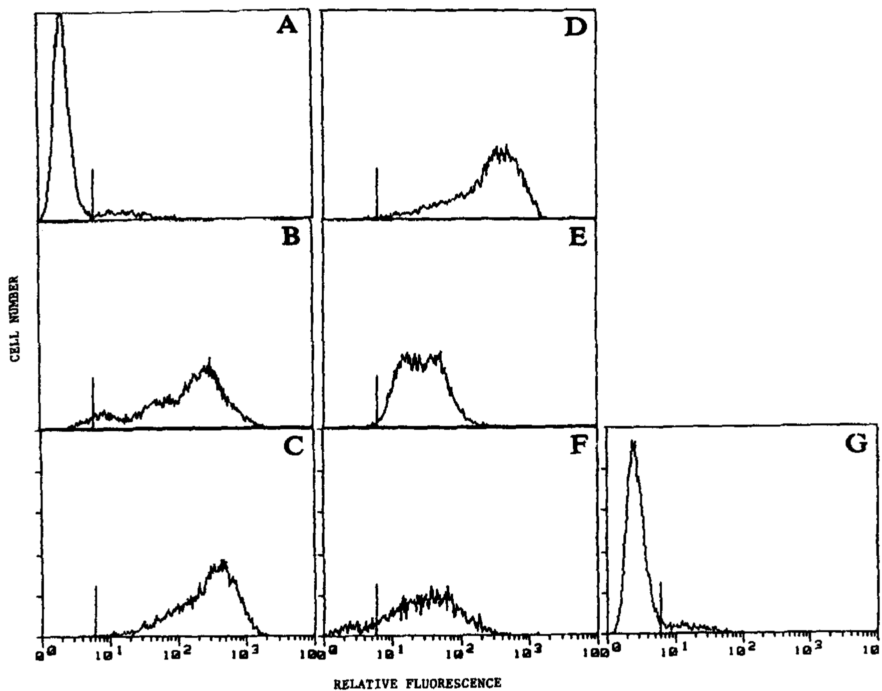


Figure 2. Comparative expression of CD44 in MC melanoma CD44 transfectants. FACS® analysis of MC melanoma transfectants showing reactivity with the Hermes-3 mAb. The cell lines were: (*A*) MC-T; (*B*) MC44E; (*C*) MC44TL; (*D*) MC44H/E; (*E*) MC44H41R-A; (*F*) MC44H; (*G*) MCα2,6ST.

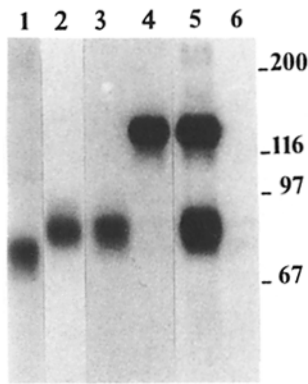


Figure 3. Immunoprecipitation of CD44 from MC melanoma CD44 transfectants. Lysates from ^{35}S-radiolabeled transfectants were immunoprecipitated with the F-10-44-2 antibody. Lysates were from: lane *1*, MC44TL; lane *2*, MC44H41R-A; lane *3*, MC44H; lane *4*, MC44E; lane *5*, MC44H/E; lane *6*, MCα2,6ST. Molecular mass markers are shown on right.

Table 2. *Comparative In Vitro Proliferation of MC Transfectants*

Cell line	[^{3}H]Thymidine incorporation
MC44E	70,190 (± 1,042)
MC44H	20,134 (± 1,050)
MC-TL	31,935 (± 665)
MC-T	80,420 (± 1,042)
MCα2,6-ST	39,276 (± 2,242)
MC41R-A	44,961 (± 1,600)
MC44H/E	39,398 (±1,304)

Thymidine incorporation of MC melanoma transfectant cell lines. All experiments were done in triplicate.

munofluorescence and immunoprecipitation (Figs. 2 and 3) whereas MC44E, and MC44H/E transfectants expressed a higher level of CD44. Control cell lines included an MC derivative expressing the neomycin resistance gene only (MC-T) and an MC line stably transfected with a β-galactoside α-2,6-sialyltransferase (MC-α2,6ST). The nomenclature used for the different transfectants and receptorglobulins is summarized in Table 1.

Phenotypic Characterization of the Melanoma Cell Transfectants. All cell lines were tested for [^{3}H]thymidine incorporation to determine their baseline growth rate (Table 2). All of the transfectants, with the exception of MC44E, had a comparable growth rate in vitro, which was lower than that of the MC-T control cell line (Table 2).

The phenotype of all transfectants was also characterized with respect to expression of adhesion molecules relevant for cell–cell and cell–ECM interaction. Thus, the parental cell line and each transfectant were assessed for β1 integrin VLA1-VLA6 expression by indirect immunofluorescence (Table 3). Comparisons between transfectants and the parental cells were made to ensure that expression of potentially relevant adhesion molecules had not been altered by the transfection itself and subsequent outgrowth under the selection conditions. The MC melanoma cell line was found to express VLA3, 5, and 6 but not VLA1, 2, and 4, and stable expression of CD44 appeared to have no significant effect on the level or

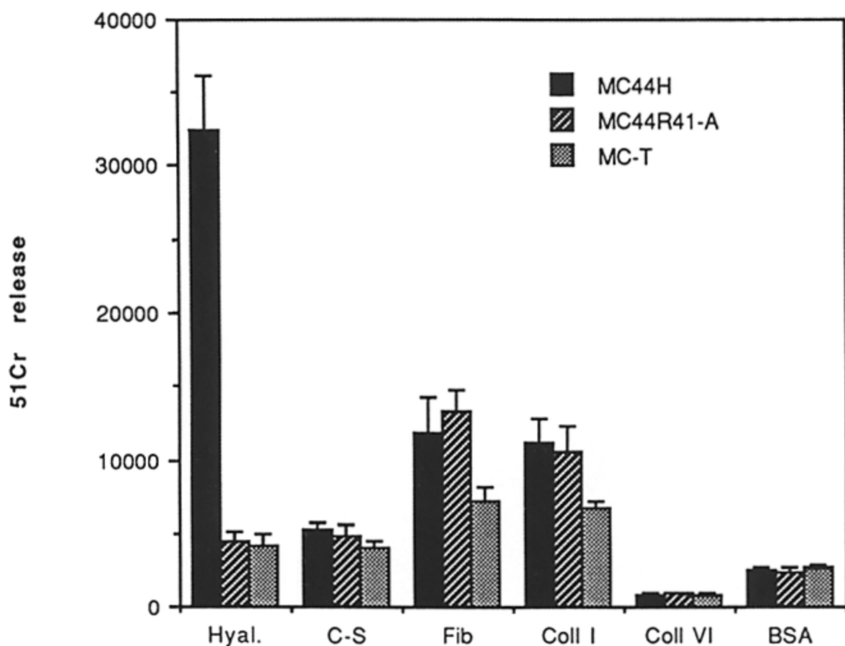
Table 1. *Characteristics of CD44 Molecules Expressed in MC Cells and Corresponding Receptorglobulins*

Transfectants	cDNA transfected/MW of corresponding cell surface CD44	Promotion of transfectant adhesion to HA coated surfaces	Receptorglobulins derived (Rg)	Binding to soluble and tissue HA
MC-T	none	− (11)		
MC44H	CD44H/85 Kd	+ + (9, 11, 23)	CD44HRg (8, 25)	+ + (8, 25)
MC-TL	CD44H cytoplasmic deletion/72 Kd	+ (11)		
MC44E	CD44E/130–150 Kd	− (9, 11, 23)	CD44ERg	+/−
MC44H/E	CD44H + CD44E	nt		
MC44H41R-A	CD44H mutant (41 Arg replaced by Ala) /85 Kd	− (25)	CD44Mut-Rg (25, and present study)	− (25, and present study)
MCα2,6ST	Human α-2,6-syalyltransferase	−		

Characteristics of CD44 molecules expressed in MC transfectants and corresponding receptorglobulins. References are shown in brackets. For transfectant adhesion to HA-coated substrate, + + and +: 6–10-fold and 2–5-fold increase of adhesion, respectively, compared with MC-T cells; −, background adhesion, typified by MC-T cells; nt, not tested.

type of β1 integrins expressed, only a minor variation in VLA5 and VLA6 expression being observed in CD44H41R-A cells (Table 3).

MC44H and MC44H41R-A Cells Differ Primarily in Their Ability to Attach to Synthetic Surface- and Tissue-bound Hyaluronate. To determine whether mutation of 41 Arg might alter CD44 interaction with molecules other than hyaluronate, that have been proposed to bind CD44 (3, 7, 18), MC44H, MC44H41R-A and MC-T cells were compared for attachment to plastic coated with hyaluronate, chondroitin sulfate, fibronectin, collagen type I, and collagen type VI. In these adhesion assays, the only significant functional difference between MC44H and MC44H41R-A cells was their capacity to attach to hyaluronate-coated surfaces (Fig. 4). Importantly, attachment of the two transfectants to collagen I and fibronectin was comparable, and only slightly greater than that of CD44-negative MC controls. In these assays, no significant CD44-mediated binding to collagen VI and chondroitin sulfate was observed. Thus, mutation of 41 Arg appears to selectively abrogate CD44-mediated MC melanoma cell attachment to hyaluronate in vitro. To determine whether the results of these assays reflect MC cell attachment in vivo, we performed adhesion experiments using rat and murine frozen tissue sections overlayed with MC transfectants. MC44H cells were observed to bind to dermis (Fig. 4), muscle and renal papilla (data not shown), whereas no significant binding of MC44H41R-A or of MC-T cells was noted (Fig. 4, and data not shown). Binding of MC44H cells could be abrogated by prior treatment of tissues with hyaluronidase (Fig. 4) but not with heparitinase or keratanase that served



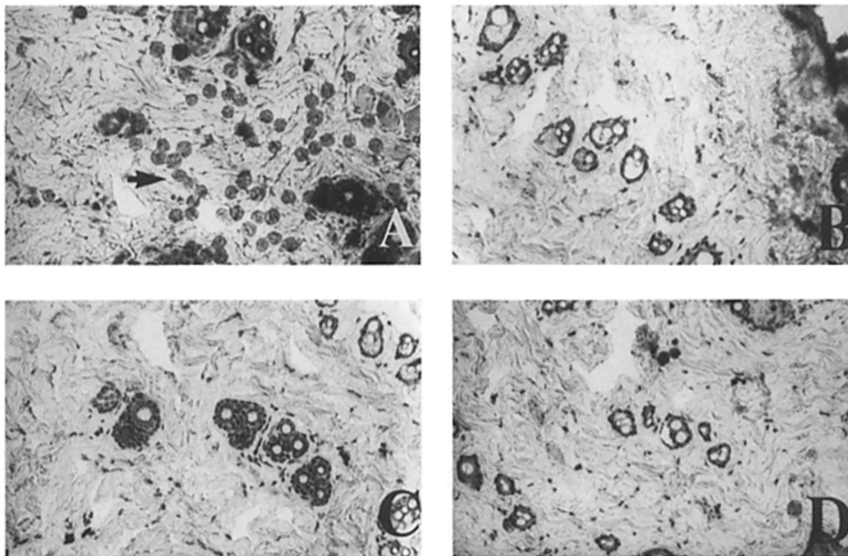


Figure 4. In vitro adhesion assays. (*A*) ^{51}Cr-radiolabeled MC-T, MC44H, and MC44H41R-A cells were seeded onto 24-well plates (at 2 × 10^5 cells/well) previously coated with HA, chondroitin sulfate, collagen I, collagen VI (each at 5 mg/ml) and fibronectin (1 mg/ml). Heat denatured BSA was used as a negative control. Adhesion assays were performed at 4°C for 30 min with gentle shaking. Nonadherent cells were washed away, adherent cells were lysed, and the released ^{51}Cr was determined. Radioactivity is expressed in cpm. All experiments were done in triplicate and standard deviations are indicated. (*B*) Adhesion of MC transfectants to frozen tissue sections. Untreated (panels *A*, *C*, and *D*) and hyaluronidase-treated (panel *B*) tissue sections of mouse skin were incubated with MC44H cells (panels *A* and *B*), MC44H41R-A cells (panel *C*), and MC-T cells (panel *D*) as described in Materials and Methods. Adherent cells are indicated by arrow. The results shown are representative of three separate experiments.

Table 3. *β1 Integrin Expression of MC Melanoma Transfectants*

Cell lines	Vla1	Vla2	Vla3	Vla4	Vla5	Vla6
MC-T	−	−	++	−	+	+
MC44H	−	−	++	−	++	++
MC44E	−	−	++	−	++	++
MC44TL	−	−	++	±	++	++
MC41R-A	±	−	++	−	+	+
MC44H/E	−	−	++	−	++	++
MCα2,6-ST	−	−	++	−	++	++

β1 integrin expression of MC melanoma transfectant cells. Integrin expression was evaluated by indirect immunofluorescence using specific anti-VLA mAb ++, homogeneous staining of 70–100% of cells; +, variable staining of 10–70% of cells; ±, staining of isolated cells (<10%).

as controls (data not shown). Thus, interaction with hyaluronate in tissues appears to be the most potent mechanism of CD44H-mediated MC cell attachment to ECM, consistent with the in vitro binding assays above.

CD44-HA Interaction Regulates the Rate of Local Tumor Growth In Vivo. To determine the effect of CD44 expression on the rate of melanoma growth in vivo, a pilot experiment was performed using three separate isolates of each transfectant (MC44H, MC44E, MC44H41R-A, MC44TL, and MC-T). An equal number of cells from each transfectant (10^7) was injected subcutaneously into one SCID mouse and the animals monitored for visible tumor growth. All three MC44H transfectants formed visible tumors within 18–21 d, whereas no tumors derived from MC44E, MC44H41R-A, and MC-T cells could be detected 30 d after injection. MC44TL transfectants formed measurable tumors within 25–30 d of injection (data not shown). This pilot experiment showed that different isolates of each transfectant formed tumors at comparable rates, and allowed the selection of a single transfectant of each type for subsequent studies.

To better evaluate the effect of expression of the different CD44 molecules on tumor development, seven groups of six SCID mice were injected subcutaneously with 6×10^6 cells/animal, each group receiving a different transfectant. The transfectants included MC-T, MCα2,6ST, MC44H, MC44E, MC44TL, MC44H41R-A, and MC44H/E. Animals were monitored three times a week for first three weeks, and then daily for calibration of tumor growth (Table 4). All animals were killed between days 36–50, when autopsy was performed, and the tumors excised and weighed. At day 25 after injection, tumors were visible in animals receiving CD44H, CD44H/E, and CD44TL transfectants (Table 4 and Fig. 5). Between days 25 and 35, a rapid increase of tumor volume was observed in animals injected with CD44H, CD44H/E, and CD44TL transfectants but not with CD44E and CD44H41R-A transfectants (Fig. 5). At day 35, the average weight of CD44H transfectant–derived tumors was 1,700 mg compared with roughly 1000 mg for H/E transfectant–derived and 800 mg for CD44TL transfectant–derived masses. In contrast, only two animals receiving MC44H41R-A ex-

Table 4. *Tumor Formation from MC Melanoma Transfectants*

Cell line	Tumor weight*		
	Day 25	day 30	Day 35
		mg	
MC44E	7 (± 1)	7 (± 1)	7 (± 1)
MC44H	17 (± 2.3)	174 (± 28)	1765 (± 302)
MC-TL	7 (± 1)	66 (± 19)	807 (± 101)
MC-T	7 (± 1)	7 (± 1)	7 (± 1)
MC-α2,6-ST	7 (± 1)	7 (± 1)	7 (± 1)
MC41R-A	7 (± 1)	7 (± 1)	83 (± 132)
MC44H/E	15 (± 14)	160 (± 90)	1070 (± 350)

Tumor formation from MC melanoma transfectants. The mean tumor size for each group (six animals/group), expressed in milligrams is indicated, along with standard deviations. 7 represents tumor size below measurable levels.

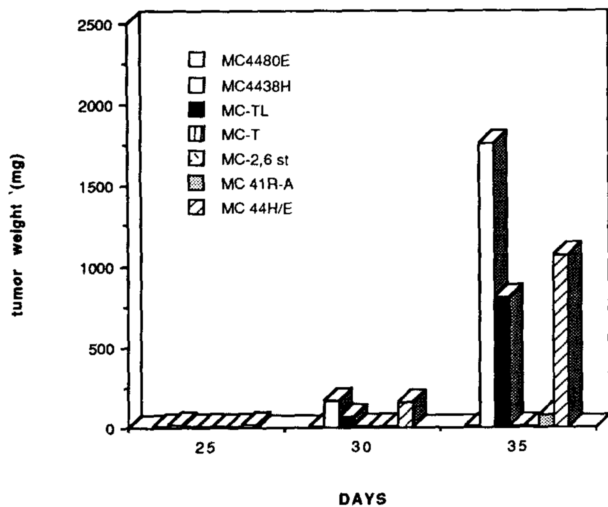


Figure 5. CD44 MC melanoma transfectant-derived tumor development in vivo. The different MC transfectants are indicated. Tumor size is expressed in milligrams. Each bar represents the mean tumor size from six animals.

pressing cells contained visible tumors at 35 d. To determine whether this tumor growth represents a significant enhancement with respect to the controls, animals receiving MC44H41R-A, MC-T, and MCα2,6ST cells were observed for a total of 50 d. Between days 45 and 50, all animals displayed tumors averaging roughly 500 mg, which reflect the intrinsic tumorigenicity of the MC melanoma. No significant difference in tumor size was observed among the three groups of animals (data not shown), indicating that expression of the CD44H mutant does not confer a tumorigenic advantage to melanoma cells. VLA expression was assessed by indirect immunofluorescence in tissue sections of explanted tumors from two animals from each group. Tumors resulting from each of the various transfectants expressed the same β1 integrins as the transfectants themselves (data not shown), indicating that in vivo growth had not altered the integrin ECM receptor repertoire of the melanoma cells. Similarly, CD44 expression was maintained in all of the transfectant-derived tumors.

CD44E Binds Hyaluronate Weakly and Is Shed from the Surface of MC Melanoma Transfectants. In previous studies using human lymphoma transfectants (9, 23) and in the present work, CD44E failed to promote cell attachment to hyaluronate in vitro or tumor development in vivo. In an attempt to determine whether this might be due to an intrinsic inability of CD44E to bind hyaluronate, we developed a CD44ERg fusion protein and examined its interaction with ECM on frozen tissue sections. CD44ERg was found to bind renal papilla interstitium weakly compared to CD44HRg, and binding could be abrogated by prior treatment of tissue with hyaluronidase (Fig. 6). On the basis of this observation, it might be expected that expression of CD44E should provide at least some tumor growth enhancement from corresponding transfectants. Furthermore, MC44H/E transfectants would be expected to form tumors at least at the same rate as CD44H expressors. The failure of CD44E to promote MC cell tumor formation and the association of its expression with partial inhibition of CD44H-dependent tumor growth enhancement in the double transfectants suggest that CD44E-mediated adhesion may be subject to inhibitory mechanisms at the cell surface. The failure to identify molecules that coprecipitate with CD44E and the observation that all known anti-CD44 mAb that recognize CD44H also recognize CD44E (25, and our unpublished observations), render remote the likelihood of interactions between CD44E and cell surface molecules that might mask or alter the conformation of the HA-binding

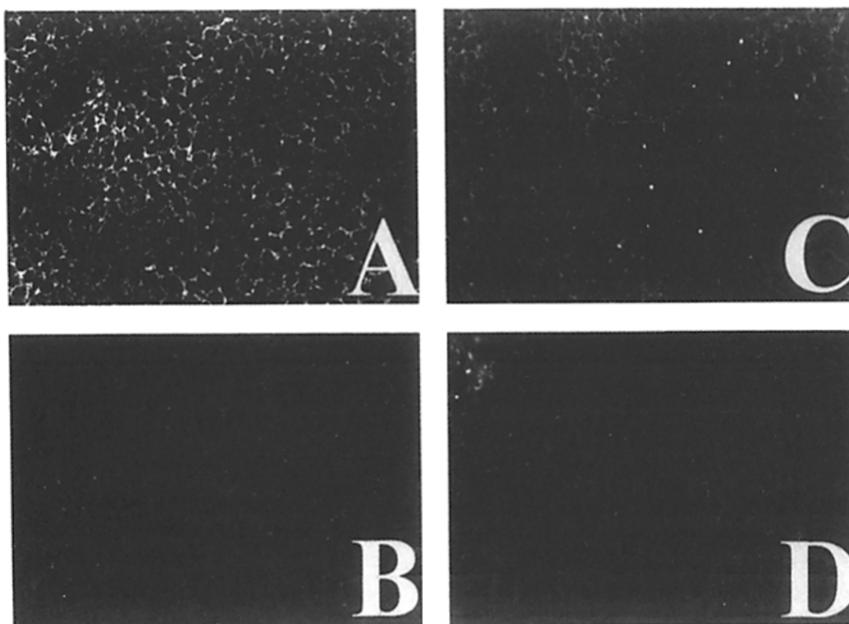


Figure 6. Binding of CD44ERg to HA in vivo. Untreated and hyaluronidase-treated frozen tissue sections of rat kidney were incubated with 10 μg/ml CD44HRg (*A* and *B*) or CD44ERg (*C* and *D*) followed by a secondary goat anti-human FITC-conjugated antibody.

domain. An alternative explanation may be shedding of CD44E from the cell surface. To test this possibility, equal numbers of MC44H, MC44E, and MC44H/E cells were [^{35}S]methionine radiolabeled and CD44 immunoprecipitated from the supernatants following a 72-h culture period. CD44E was found to be massively shed from MC transfectants as opposed to only slight shedding of CD44H (Fig. 7). Thus, the weak binding of CD44E to hyaluronan coupled to its shedding from the cell surface may explain the inability of CD44E to promote MC-derived tumor development. The massive shedding of the E isoform may partly inhibit CD44H-mediated attachment to ECM, providing a possible explanation for the intermediate growth rate of MC44H/E tumors.

A Strategy for Testing the Effect of Wild-type and Mutant Soluble Recombinant CD44 on Local Melanoma Growth. The above results suggest that binding of CD44 to hyaluronate plays a critical role in CD44-mediated enhancement of local tumor growth. Because hyaluronate in solid tissues is bound to ECM proteoglycans, the validity of this observation can be tested by comparing the effect of soluble CD44HRg and CD44MutRg on local tumor formation of exogenously administered CD44H-expressing melanoma cells. Based on the tissue-binding capacity of CD44HRg, and the observation that CD44HRg can block hematogenous spread of CD44H-expressing lymphomas (28), it would seem reasonable to expect that CD44HRg might compete with cell surface CD44H for hyaluronate binding, thereby possibly inhibiting tumor growth. CD44MutRg, on the other hand, should have no effect. Previous work had indicated that CD44Rg might have a short half-life in vivo (28), suggesting that any functional study is likely to require repeated and frequent administration of the fusion protein. Because repeated daily local injections of soluble CD44 would represent a technical difficulty in determining the exact tumor cell injection site, in addition to causing animal discomfort, 100 μl osmotic pumps containing CD44HRg, CD44MutRg, or human IgG were implanted subcutaneously at the site of subsequent tumor cell injection. These pumps administer their contents by continuous infusion over a period of 7 d, and the injected medium diffuses into the subcutaneous tissues in the vicinity of the pump. To be optimally exploited, this strategy would require the use of CD44H-expressing, hyaluronate-binding cells which rapidly form tumors in vivo, so that the effect of the receptorglobulins might readily be determined. To this end, the murine melanoma B16F10 was selected.

Characterization of Murine Melanoma CD44 Expression and Function. The use of the murine B16F10 melanoma offers several advantages. First, B16F10 cells display an aggressive growth pattern in nude mice, allowing rapid assessment of the effect of CD44 receptorglobulins. Second, B16-derived tumors are pigmented and their local spread can be readily visualized without resorting to surgical procedures. Third, if CD44HRg were to prove effective in blocking tumor formation in this model, it would provide additional support for the importance of CD44-HA interaction, and suggest

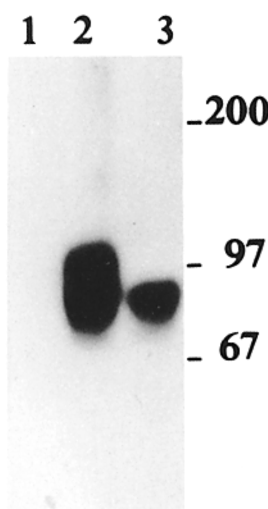


Figure 8. CD44 expression by B16F10 melanoma cells. Immunoprecipitation of CD44 from ^{35}S-radiolabeled cell lysates was performed using the IM7 mAb. Lysates were from: lane *1*, MC-T; lane *2*, B16F10; lane *3*, MC-CD44H. Molecular mass markers are shown on right.

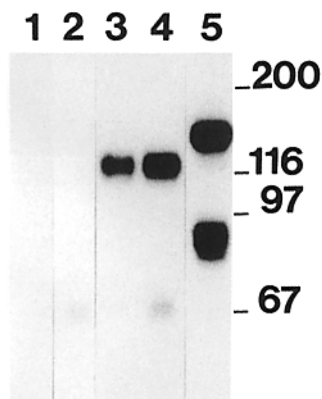


Figure 7. Shedding of CD44 from the surface of MC transfectants. Equal numbers of MC44H, MC-T, MC44E, and MC44H/E cells were ^{35}S-radiolabeled and culture supernatants harvested 72 h after labeling. CD44 was immunoprecipitated from the supernatants using anti-CD44 mAb and protein A–Sepharose beads. Supernatants were from: lane *1*, MC-T cells; lane *2*, MC44H cells; lane *3*, MC44E cells; lane *4* MC44H/E cells; lane *5*: immunoprecipitation of cell surface CD44H and E from MC44H/E transfectants. Molecular mass markers (kD) are indicated.

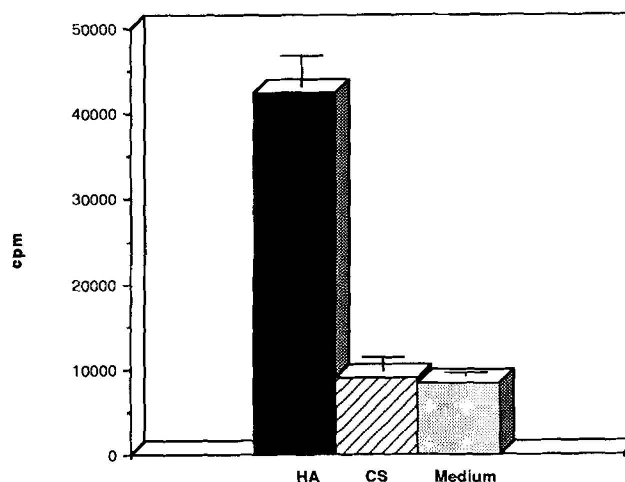
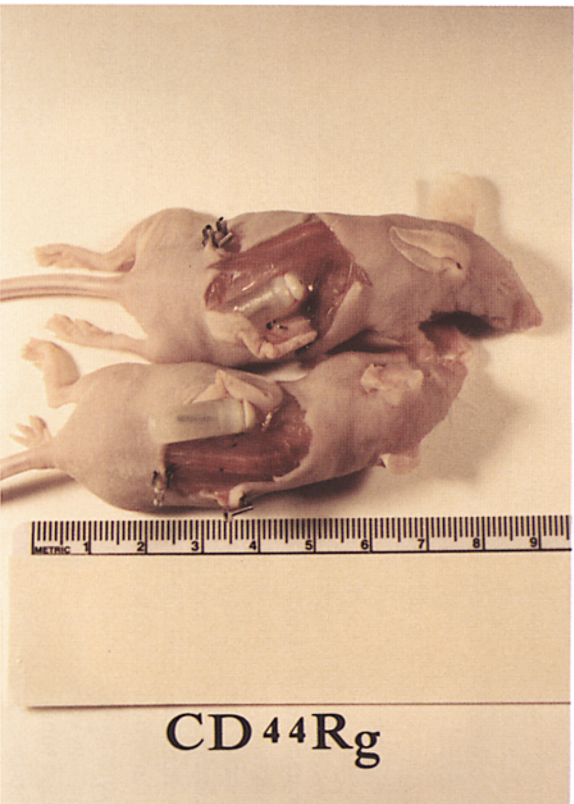


Figure 9. Binding of B16 melanoma cells to HA. Binding to HA-, chondroitin sulfate-, and PBS-coated wells is expressed as ^{51}Cr radiolabel released from adherent cells after lysis by detergent. All experiments were done in quadruplicate.



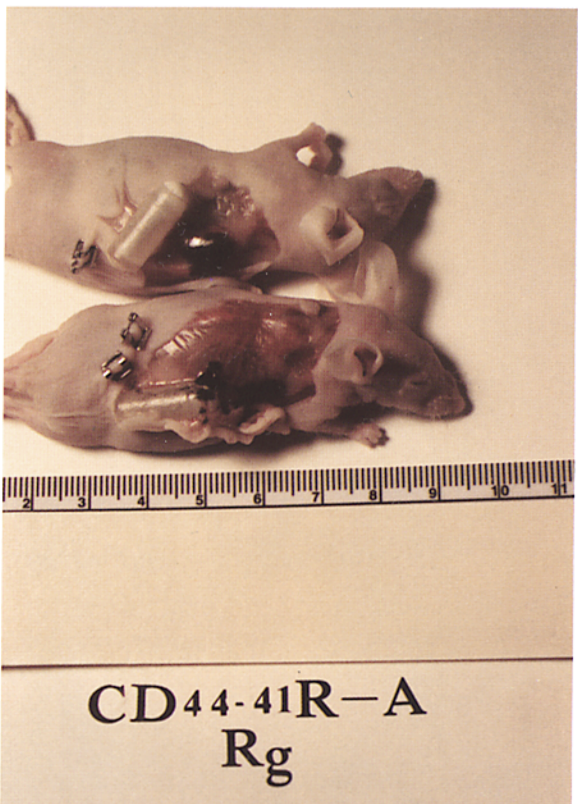


Figure 10. Blocking of B16F10-derived melanoma growth by soluble CD44Rg. Mice were killed 8 d after tumor injection and 1 d after completion of soluble molecule delivery by osmotic pumps. The pumps contained: (*A*) human IgG; (*B*) CD44HRg; (*C*) CD44H41R-ARg. Ten animals were used in each group. Two representative animals from each group are shown.

that any potential species-specific CD44-ligand interactions might only play a minor role in promoting tumor development. B16F10 cells tested positive for reactivity with the anti-CD44 mAb IM7, and immunoprecipitation of B16F10 lysates with IM7 revealed an 85-kD isoform, corresponding to CD44H, and an additional 100-kD species (Fig. 8). The cells were observed to adhere to HA-coated surfaces (Fig. 9).

Local Infusion of CD44HRg Blocks Tumor Growth. An initial pilot study was performed to determine whether infusion of CD44HRg had any blocking effect at all on local tumor growth. Four animals were injected with 5×10^6 B16 melanoma cells each, in the immediate vicinity of the implanted pump. Two of the animals had pumps containing human IgG at 1.5 mg/ml, while two other mice had similar pumps containing CD44HRg at 1.5 mg/ml. At the end of 7 d both sets of animals showed tumor growth, but the tumors in animals bearing CD44HRg-infusing pumps were significantly smaller than their counterparts in animals infused with IgG (data not shown). Because the B16 melanoma grows rapidly in vivo, it seemed likely that the amount of CD44HRg infused might be insufficient to block tumor development from such a large number of injected cells. To optimize the conditions, the experiments were repeated by injecting each animal with 125×10^3 cells. Three groups of ten animals were used, carrying pumps containing 0.1 ml of either human IgG, CD44HRg, or CD44MutRg, each at 6 mg/ml. 8 d after tumor injection, and 1 d after the cessation of immunoglobulin and receptorglobulin delivery by the osmotic pumps, eight animals from each group were killed and tumor growth assessed. Whereas both groups of animals receiving human IgG and CD44MutRg revealed large tumor masses (1–2 cm in diameter), no tumor formation was observed in mice infused with CD44HRg (Fig. 10). Two animals from each group were followed for an additional week in the absence of immunoglobulin or receptorglobulin administration. Although tumor masses appeared in the animals which had previously received CD44HRg, they measured 2–3 mm compared with 3–4 cm for tumors derived from animals that had been infused with IgG and CD44MutRg (data not shown). Additional control experiments were performed by injecting animals with the same number of B16 melanoma cells in 0.1 ml of a 6-mg/ml solution of CD44Rg or human IgG, but without continuous CD44Rg infusion. Although the presence of CD44Rg at the time of injection significantly slowed down the tumor growth with respect to IgG, tumors were visible 3 d after injection and had grown to a diameter of 0.5–0.8 cm by day 8 (data not shown). Continuous infusion of CD44Rg therefore appears necessary to effectively block local melanoma growth. Tumor growth inhibition by CD44Rg was not due to a direct effect on the melanoma cells since B16 melanomas cultured in the presence of 6 mg/ml CD44Rg did not show a significant modification in their growth rate (Table 5).

Table 5. *Proliferation Assay of Murine Melanoma B16 Cells*

CD44HRg	Human IgG	Standard medium
18,479 ± 928	20,253 ± 1,377	20,435 ± 1,861

Thymidine incorporation of B16F10 cells growth in the presence of CD44HRg and human IgG. All experiments were done in triplicate.

Discussion

HA has been observed at sites of tumor invasion (29) and several studies have suggested that HA might in some way be associated with tumor aggressiveness (30, 31). This notion is supported by observations that high expression of the HA receptor, CD44, correlates with aggressiveness of lymphoid tumors (32) and invasiveness of bladder carcinomas (31). Our own studies have demonstrated that expression of an HA binding form of CD44 (CD44H) in a human lymphoma significantly augments the rate of local tumor development in vivo as well as the rate of tumor formation in various organs as a result of hematogenous dissemination (23). Tumor development resulting from hematogenous dissemination of CD44H-expressing lymphoma cells could be blocked by soluble CD44Rg (28). The choice of melanomas for the present experiments was twofold. First, most melanomas express high levels of CD44H (13, 33, 34), which might regulate their dermal penetration and intradermal growth, especially since dermis is abundant in HA (35). Assessment of the role of CD44 expression in melanoma growth may therefore have direct biologic and clinical relevance. Second, previous experiments on the role of CD44 in tumor growth promotion were performed using lymphoma cells, and it seems critical to determine whether CD44 isoforms behave in a similar way in cells of different origin or whether their impact on tumor growth varies according to the cell type in which they are expressed. Melanomas, being of neuroectodermal origin, provide a suitable counterpart to mesoderm-derived lymphoid cells. The present study provides clear evidence that the two CD44 isoforms tested, CD44H and CD44E behave in a similar manner in melanomas and in lymphomas with regard to the regulation of tumor growth in vivo.

Interestingly, expression of CD44H cytoplasmic tail deletion mutants significantly promotes tumor growth although not to the same degree as wild-type CD44H. This is consistent with the observations that cells expressing CD44H cytoplasmic deletion mutants can still adhere to hyaluronate-coated surfaces but with significantly lower efficiency than wild-type CD44H-expressing transfectants (11, 36). The cytoplasmic domain of CD44 is thought to interact with the cytoskeleton (37–39) and its presence is required for cell migration on HA-coated substrates (11). Expression of the cytoplasmic tail may also stabilize the cell surface conformation of CD44 and/or facilitate its aggregation on the cell surface which is proposed to be required for optimal HA binding (40, 41). However, the present observations clearly suggest that biologically significant interaction between cell surface CD44H and ECM-bound HA can occur in the absence of the cytoplasmic tail.

Similar to lymphoma transfectants, CD44E-expressing

melanoma cells were not observed to display enhanced tumor formation with respect to controls. We have shown here that a CD44ERg fusion protein binds HA in tissues, although not as strongly as CD44HRg, supporting the notion that the presence of variably spliced exons may influence HA binding ability of CD44. Furthermore, CD44E was observed to be spontaneously shed from the surface of MC transfectants, providing a potential explanation for its inability to promote MC-derived tumor development, and partial inhibition of CD44H-dependent MC44H/E tumor growth. These observations raise two interesting possibilities. The first is that selective shedding of CD44 isoforms may play a role in the regulation of CD44-associated cell–substrate interactions. The second is that shedding may provide a cell type–specific selection pressure for CD44 splice variant expression, since CD44 is not observed to be shed from several human tumor cell lines that constitutively express CD44E (Bartolazzi, A., and I. Stamenkovic, unpublished observations). Elucidation of mechanisms that determine preferential shedding of one CD44 isoform with respect to another will require further study.

The principal finding in the present work is that CD44H–HA interaction provides an important mechanism by which CD44H-mediated promotion of tumor growth is regulated in vivo. This observation is based on the use of a recombinant CD44H molecule containing a point mutation that changes an arginine residue at position 41 of the extracellular domain to alanine (25). The cell surface–expressed CD44H mutant does not bind soluble (Peach, R., and A. Aruffo, unpublished observations) or surface-bound HA and melanoma cell transfectants expressing the CD44H mutant fail to show the rapid tumor formation in SCID mice characterized by wild-type CD44H-expressing counterparts. In our assays, transfectants expressing the mutant CD44 molecule and MC44H cells bound comparably to a variety of substrates that have been advocated as CD44-binding molecules. Thus, it would appear that the point mutation of 41 Arg in the melanoma model presented here does not alter other known CD44 adhesive properties. It would also appear unlikely that the point mutation results in a CD44 signaling defect, since MC44TL cells, which express a cytoplasmic deletion mutant that cannot promote cell migration on hyaluronan, and therefore appropriate intracellular signaling, form tumors more rapidly than parental and CD44H41R-A cells. Although we cannot exclude the possible participation of another, as yet unidentified, CD44 ligand, these observations strongly support the notion that communication between the extracellular domain of CD44H and its principal ligand, HA, is critical for CD44-dependent augmentation of tumor growth.

CD44H expression may therefore provide a general pathway for the regulation of tumor development regardless of the histologic nature of the tumor. However, it has recently been demonstrated that CD44H on normal murine leukocytes does not bind HA with high affinity, and that leukocyte activation is required for high affinity binding to occur (42). This observation suggests that leukocyte activation may result in a conformational change of CD44H that augments its HA binding affinity. In several human tumors that we have tested, CD44H appears to be constitutively expressed in an HA-binding conformation (9, 11, 34, Bartolazzi, A., and I. Stamenkovic, unpublished observations), but there are reports that some tumors expressing this isoform do not bind HA (33). Assessment of the HA-binding capability that CD44H confers to tumor cells may therefore be a superior indicator of potential tumor aggressiveness than CD44H expression alone.

We had previously shown, using a lymphoma model, that soluble CD44Rg could effectively inhibit hematogenous dissemination of CD44H-expressing, HA-binding lymphoid tumor cells (28). In the present study, CD44HRg is observed to block local tumor development from highly tumorigenic melanoma cells, and underscores the critical nature of CD44H-HA interaction, since CD44MutRg has no inhibitory effect on B16 melanoma growth. Optimal blocking of tumor growth appears to require continuous infusion of soluble CD44HRg. This may be due in part to the short half-life of the molecule in vivo (28), but may probably also be attributed to the high turnover rate of HA (35). In addition to confirming the importance of CD44H-HA interaction in regulating the rate of tumor formation, these observations suggest that local infusion of CD44HRg might provide a potential approach to helping contain regional tumor spread.

Recent work has suggested that a distinct CD44 isoform might play an important role in determining the metastatic proclivity of an adenocarcinoma in rats (16). However, another study has shown that expression of the corresponding human homolog is downregulated in human squamous carcinoma metastases (43). Whether the metastasis-promoting effect of this isoform in rat adenocarcinoma cells is due to its interaction with HA or other putative ligands in host tissues remains to be determined. It is possible, for example, that certain tumor cells might induce post-translational modifications of variable exon-encoded sequences that facilitate interactions with ligands other than HA and that may influence metastatic tumor cell behavior. This view is supported by studies showing that addition of chondroitin sulfate side chains to CD44 can promote its interaction with fibronectin (18).

In addition to HA and fibronectin, CD44H has been observed to display affinity for chondroitin sulfate (8, 9). Whereas recognition of some of the alternative ligands by CD44H may have potential physiologic relevance in certain cellular interactions (3), the present study suggests that interaction with ECM-bound HA constitutes a critical step in CD44H-mediated promotion of tumor growth. The precise mechanism by which enhancement of tumor formation might occur is unclear. One possibility is that HA may trigger CD44-mediated signals, a view that is supported by studies showing that antibody stimulation of CD44 augments cytokine production by monocytes (44), and that macrophage interaction with HA may result in growth factor secretion (45). An alternative view might be that CD44H participates in HA degradation (46), which might facilitate tumor cell invasion of host

tissues. The observation that tumor cells expressing CD44 cytoplasmic deletion mutants form tumors more rapidly than CD44-negative controls would seem to support the latter possibility. In the simplest scenario, consistent with our previous and present results, HA might serve as a molecular bridge, promoting tumor cell interaction with host tissue stroma, which is critical to both primary and metastatic tumor growth.

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