Molecular Biology of the Cell
Vol. 7, 383–396, March 1996

CD44/Chondroitin Sulfate Proteoglycan and α2β1 Integrin Mediate Human Melanoma Cell Migration on Type IV Collagen and Invasion of Basement Membranes

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Submitted April 18, 1995; Accepted December 15, 1995
Monitoring Editor: Richard Hynes

Tumor cell invasion of basement membranes (BM) represents one of the critical steps in the metastatic process. Tumor cell recognition of individual BM matrix components may involve individual cell adhesion receptors, such as integrins or cell surface proteoglycans, or may involve a coordinate action of both types of receptors. In this study, we have focused on the identification of a cell surface CD44/chondroitin sulfate proteoglycan (CSPG) and α2β1 integrin on human melanoma cells that are both directly involved in the in vitro invasion of reconstituted BM via a type IV collagen-dependent mechanism. Interfering with cell surface expression of human melanoma CSPG with either *p*-nitrophenyl-β-D-xylopyranoside treatment or anti-CD44 monoclonal antibody (mAb) preincubation inhibits melanoma cell invasion through reconstituted BM. These treatments also strongly inhibit melanoma cell migration on type IV collagen, however, they are ineffective at inhibiting cell adhesion to type IV collagen. Purified melanoma cell surface CD44/CSPG, or purified chondroitin sulfate, bind to type IV collagen affinity columns, consistent with a role for CD44/CSPG-type IV collagen interactions in mediating tumor cell invasion. In contrast, melanoma cell migration on laminin (LM) does not involve CD44/CSPG, nor does CD44/CSPG bind to LM, suggesting that CD44/CSPG-type IV collagen interactions are specific in nature. Additionally, anti-α2 and anti-β1 integrin mAbs are capable of blocking melanoma cell invasion of reconstituted BM. Both of these anti-integrin mAbs inhibit melanoma cell adhesion and migration on type IV collagen, whereas only anti-β1 mAb inhibits cell adhesion to LM. Collectively, these results indicate that melanoma cell adhesion to type IV collagen is an important consideration in invasion of reconstituted BM in vitro, and suggest that CD44/CSPG and α2β1 integrin may collaborate to promote human melanoma cell adhesion, migration, and invasion in vivo.

INTRODUCTION

Tumor cell invasion is a complex process, involving tumor cell adhesion, migration, and the dissolution of intact extracellular matrix (ECM) molecules (Stetler-Stevenson *et al.*, 1993). Tumor cell invasion through basement membranes (BM)[1] is essential for the development of metastatic foci. BM are thin layers of spe-

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[1] Abbreviations used: BM, basement membranes; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; ECM, extracellular matrix; GAG, glycosaminoglycans; LM, laminin; mAb, monoclonal antibody; MPG/NG2, melanoma-associated proteoglycan; PBS, phosphate-buffered saline; PG, proteoglycans.

cialized ECM that are composed of several constituents, including type IV collagen, laminin (LM), entactin/nidogen, and heparan sulfate proteoglycans (Paulsson, 1992). Although incompletely understood, there is substantial evidence indicating that these, and other, BM components can modulate tumor cell adhesion and motility, and as a result could influence metastasis formation. The complexity of tumor cell recognition of BM components is illustrated, in part, by the multiple cellular adhesion sites that have been identified on proteins such as type IV collagen and LM. Multiple, nonoverlapping proteolytic fragments (Chelberg *et al.*, 1989; Cameron *et al.*, 1991; Perris *et al.*, 1993; Karecla *et al.*, 1994; Lallier *et al.*, 1994; Matter and Laurie, 1994; Simon-Assmann *et al.*, 1994) or synthetic peptides derived from BM components (Furcht *et al.*, 1984; Chelberg *et al.*, 1990; Cameron *et al.*, 1991; Wilke and Skubitz, 1991; Gehlsen *et al.*, 1992b; Fields *et al.*, 1993; Nomizu *et al.*, 1993) have been identified that support both normal and transformed cell adhesion. The importance of BM proteins to the process of tumor cell invasion and/or metastasis is emphasized by the ability of adhesion-disrupting proteolytic fragments or synthetic peptides from these proteins to inhibit tumor cell invasion in vitro (Kanemoto *et al.*, 1991) or metastasis formation in vivo (Iwamoto *et al.*, 1987; Nakai *et al.*, 1992; Nomizu *et al.*, 1993; Yamamura *et al.*, 1993; Kawasaki *et al.*, 1994; Zhao *et al.*, 1994).

Tumor cells express several receptors that can interact with BM components and influence tumor cell adhesion, migration, and/or invasion. For example, several members of the integrin family, including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$, can interact with BM components (Elices and Hemler, 1989; Yamada *et al.*, 1990; Mortarini *et al.*, 1991; Chan *et al.*, 1992; Chan and Hemler, 1993; Etoh *et al.*, 1992, 1993; Bosman, 1993; Dedhar *et al.*, 1993; Yoshinaga *et al.*, 1993; Paulus and Tonn, 1994; Melchiori *et al.*, 1995). Furthermore, $\alpha 6\beta 4$ integrin (Dedhar *et al.*, 1993) and $\alpha v\beta 3$ integrin (Gehlsen *et al.*, 1992a; Seftor *et al.*, 1993) have been implicated in modulating the attachment and/or invasion of certain carcinomas and melanomas, respectively. With specific regard to interaction with BM proteins, increased $\alpha 2\beta 1$ integrin expression has been shown to not only increase adhesion to collagen and LM but also to enhance both experimental and spontaneous metastasis of rhabdomyosarcoma cells (Chan *et al.*, 1991).

Despite the importance of integrins, there is also an important role for cell surface proteoglycans (PG) in modulating the adhesion and/or migration of normal and transformed cells (see reviews by Faassen *et al.*, 1992a; Gallagher, 1989). Cell surface PG consist of plasma membrane–associated core proteins that are modified by the covalent addition of glycosaminoglycans (GAG) (Ruoslahti, 1988; Gallagher, 1989). Although melanoma cells can express both heparan sulfate and chondroitin sulfate (CS) (Bumol and Reisfeld, 1982; Harper and Reisfeld, 1983; Lynch *et al.*, 1991; Moczar *et al.*, 1993), selective removal of CS inhibits melanoma cell migration and invasive behavior on type I collagen gels (Faassen *et al.*, 1992b, 1993). There have been several types of core proteins identified as potential cell surface PG, consistent with the diverse roles played by this group of molecules in modulating cell behavior (Gallagher, 1989; Faassen *et al.*, 1992a). With respect to human melanoma cells, the two most characterized cell surface PG core proteins are CD44 and melanoma-associated PG, MPG/NG2, both of which are expressed on this cell type as chondroitin sulfate proteoglycan (CSPG) (Bumol and Reisfeld, 1982; Harper and Reisfeld, 1983; Spiro *et al.*, 1991; see review by Lesley *et al.*, 1993). Both of these PG have been implicated in modulating melanoma cell adhesion or motility on various ECM components or to other cells (see reviews by Faassen *et al.*, 1992a; Lesley *et al.*, 1993).

The current studies were performed to evaluate a potential role for melanoma cell surface CSPG in modulating human melanoma cell adhesion and migration on purified BM components and invasive behavior through reconstituted BM in vitro. Several lines of evidence demonstrate that CD44/CSPG plays an important role in mediating human melanoma cell migration on type IV collagen, but not LM. Furthermore, this melanoma CSPG is important for the invasive behavior of human melanoma cells, because removal of cell surface CS or anti-CD44 monoclonal antibodies (mAbs) can inhibit melanoma cell invasion through reconstituted BM. Interference with the type IV collagen integrin, $\alpha 2\beta 1$, with anti-integrin subunit mAbs can also specifically inhibit human melanoma cell migration and adhesion to type IV collagen and invasion of reconstituted BM. Collectively, the results from this study demonstrate an involvement of both cell surface CD44/CSPG and $\alpha 2\beta 1$ integrin in the promotion of human melanoma invasion of BM through a type IV collagen-dependent mechanism.

MATERIALS AND METHODS

Cell Culture

The human melanoma cell line, SK-MEL-2, was purchased from the American Type Culture Collection (Rockville, MD) and the human melanoma cell line, M14#5, was a gift from Dr. Barbara Mueller (The Scripps Research Institute, La Jolla, CA). Both cell lines were maintained in MEM (Celox, Hopkins, MN) supplemented with 10% fetal calf serum and sodium pyruvate (Life Technologies, Grand Island, NY). The number of in vitro passages was limited to eight to minimize phenotypic drift.

mAbs

mAbs used in these studies included the following: anti-CD44, Hermes-3 (mouse ascites; Jalkanen *et al.*, 1987) kindly provided by Dr. Eugene Butcher (Department of Pathology, Stanford University

School of Medicine, Stanford, CA); anti-MPG/NG2, 9.2.27 (mouse purified IgG; Bumol and Reisfeld, 1982) kindly provided by Dr. Ralph A. Reisfeld (Scripps Institute); and anti-β1 (purified P5D2 IgG; kindly provided by Dr. Leo Furcht, Department of Laboratory Medicine and Pathology, University of Minnesota). The mAbs, anti-α2 (mouse ascites, clone P1E6; Wayner *et al.*, 1988), anti-α5 (mouse ascites, clone P1D6; Wayner *et al.*, 1988), and the anti-CD44 mAb, HAR 1968 (mouse ascites, clone P1G12; Gallatin *et al.*, 1989) were purchased from Chemicon International (Temecula, CA). The anti-integrin subunit mAb, anti-α3 (mouse ascites, clone P1B5; Wayner and Carter, 1987) was purchased from Life Technologies (Gaithersburg, MD). Human melanoma cells were preincubated with either a 1:200 dilution of mAb ascites or 5 μg/ml of purified IgG (or normal mouse ascites or IgG) for 20 min at 37°C before adding cells to corresponding assays.

Invasion Assays

Invasive abilities of the human melanoma cell lines were assessed as follows: Matrigel BM matrix invasion chambers (Collaborative Biomedical Products, Bedford, MA) were removed from 4°C storage and allowed to come to room temperature. MEM (200 μl/well) was then added to each cell culture insert to rehydrate the matrigel-coated filter for 90 min at 37°C. MEM supplemented with 50 μg/ml fibronectin (200 μl/well) was added to the space below the cell culture inserts to facilitate cell attachment to the underneath surface of the filter. Cells were released with trypsin (0.25%)/EDTA (1 mM), washed, and resuspended in MEM supplemented with 10% fetal bovine serum at a cell concentration of 2×10^5 cells/ml and appropriate concentrations of inhibitors for various assays. A cell suspension of 3×10^4 cells (200 μl/well) was then added to each well and the chamber was incubated at 37°C for 18–20 h. The filter surfaces of the cell culture inserts were fixed and then stained with Diff-Quik staining solutions (Baxter Scientific Products, McGraw Park, IL). The upper surface of each filter was wiped dry with a cotton swab to remove cells that were unable to invade and then removed from the culture insert with a razor blade. Filters were mounted on slides and quantitated microscopically (15 fields per filter at a 10× objective magnification, 2× optovar setting on a Zeiss universal microscope; Oberkochen, West Germany). Each assay condition was performed in triplicate and results are expressed as an average of the triplicates ± SEM. Cellular viability (>95%) was determined by trypan blue exclusion following the termination of each assay.

Adhesion and Migration Assays

Cell adhesion was assessed in protein-coated 96-well Immulon-1 plates (Dynatech Laboratories, Chantilly, VA) as described previously (Faassen *et al.*, 1992b). Triplicate wells were precoated with a 10 μg/ml concentration of proteins overnight at 37°C and blocked with 1× phosphate-buffered saline (PBS)/0.5% bovine serum albumin for 2 h at 37°C to prevent nonspecific binding. Cell cultures were radiolabeled with 2 μCi/ml [^{3}H]thymidine (New England Nuclear, Boston, MA; specific activity: 6.7 Ci/mmol) for 18 h before performing adhesion assays. Cells were released with 1 mM EDTA/PBS, washed, and resuspended to a final concentration of 5×10^4 cells/ml in adhesion medium (MEM supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 5 mg/ml bovine serum albumin). A cell suspension of 5×10^3 cells/well was added in 100 μl of medium and allowed to adhere to protein-coated wells for 15–30 min. Following a series of mild washes, adherent cells were lysed in 150 μl of 0.5 N NaOH containing 1% SDS, and radioactivity was quantitated with a Beckman LS6500 liquid scintillation counter. Results are expressed as a percentage of the total number of input cells ± SEM.

Migration assays were assessed in modified Boyden chambers (Neuroprobe, Bethesda, MD) with 8-μm pore size polyvinylpyrrolidone-free polycarbonate filters (Nucleopore, Pleasanton, CA). The underneath surfaces of the filters were precoated with either type IV collagen or LM at a concentration of 10 μg/ml as described previously (Faassen *et al.*, 1992b, 1993). Cells were released as described above and resuspended in MEM supplemented with 20 mM HEPES at a final concentration of 4×10^5 cells/ml. Lower wells of the chambers were filled with MEM supplemented with 20 mM HEPES and appropriate concentrations of *p*-nitrophenyl-α-/β-D-xylopyranoside (α-/β-D-xyloside) or mAbs in corresponding assays. Cells were added to the upper wells with appropriate concentrations of inhibitors at 2×10^4 cells/well and incubated for 4 h at 37°C. Filters were then removed from the chambers, fixed, and stained with Diff-Quik staining solutions, mounted on slides, and migrated cell numbers were quantitated with an Image Analysis System (Optomax, Hollis, NH). Data are expressed as the number of migrated cells per square millimeter ± SEM.

α-D-Xyloside and β-D-Xyloside Pretreatment

To assess the effect(s) of β-D-xyloside treatment on cellular behavior and expression of cell surface receptors, melanoma cells were treated with these inhibitors as described previously (Faassen *et al.*, 1992b). Briefly, cells were exposed to α-/β-D-xyloside for 24 h to inhibit CSPG synthesis by replacing the medium of cell cultures (~60% confluency) with MEM containing 2.5% fetal bovine serum, with or without 1 mM β-D-xyloside (Sigma Chemical, St. Louis, MO) or α-D-xyloside (NBS Biologicals, Hatfield, UK). α-D-Xyloside, an inactive analogue of β-D-xyloside, was used as a control in these studies. A concentration of 1 mM α- or β-D-xyloside was also included in the medium throughout the assays. Inhibitory effects were reversible and protein synthesis was not affected at this concentration. Cellular viability (>95%) was determined by trypan blue exclusion.

Flow Cytometry

Cells were harvested as described above and washed three times with RPMI 1640 (Celox) containing 1% heat-inactivated goat serum, 20 mM HEPES, and 0.02% NaN_3 (FACS buffer). Cell pellets were then resuspended in FACS buffer at a concentration of 10^5 cells/ml with 10 μg/ml normal goat IgG for 30 min at 4°C and then washed three times with FACS buffer. An aliquot of 1 ml cell suspension was then incubated with 5 μg/ml (purified IgG) or a 1:200 dilution (ascites) of mAb for 30 min at 4°C with tapping periodically to disperse cells. Cells were washed three times with FACS buffer and then incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Organon Teknika, West Chester, PA; final dilution of 1:500) at 4°C for 40 min with tapping periodically to disperse cells. Antibody titers of both primary and secondary mAb were maximized under these experimental conditions. Cells were then washed three times with FACS buffer and resuspended in 300 μl of PBS containing 2% formaldehyde. Flow cytometric analyses were then performed on a FACS Star System (Becton Dickinson, Mountain View, CA).

Proteoglycan Extraction and High Performance Liquid Chromatography (HPLC) Purification of Cell Surface CSPG

Melanoma cell surface CSPG were extracted, purified, and partially characterized according to the method of Faassen *et al.*, 1992b. Briefly, melanoma cell PG were preferentially labeled with ^{35}S-sulfate for 18 h by replacing the medium in ~80% confluent cultures with low sulfate RPMI 1640 containing 50 μCi/ml $Na_2{}^{35}SO_4$ (Carrier free; ICN Biomedicals, Irvine, CA; 25.6 mCi/0.029 ml). Detergent-extracted ^{35}S-PG were dialyzed into DEAE buffer (0.15 M Tris, 6.0 M Urea, O.1 M NaCl, 0.01 M EDTA, 0.01 M 6-aminohexanoic acid, 0.2% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.0) and purified by HPLC (Beckman Model 110A) with a 7.5 × 75 mm TSK DEAE 5PW anion exchange column (Bio-Rad Laboratories, Richmond, CA) using a linear salt gradient from 0.1 to 0.8 M

NaCl over a 45-min period. The salt gradient was monitored by conductivity measurements using a Radiometer Conductivity Meter (model CDM 83). Calculations of the actual salt concentrations of each fraction were then made by comparisons to standards of known NaCl concentration in DEAE buffer. Recovery of radioactivity from the HPLC-DEAE columns was >90%.

Type IV Collagen and LM Affinity Chromatography

Type IV collagen and LM were kindly provided by Dr. Leo Furcht (Department of Laboratory Medicine and Pathology, University of Minnesota). Proteins were coupled to activated CH-Sepharose 4B according to the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, NJ). Mock-coupled columns were also prepared without proteins for use as control columns to remove nonspecific binding to the matrix. A concentration of 10 mg protein/ml of final gel volume was dissolved in coupling buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate, pH 8.6), added to preswollen beads, and incubated overnight at 4°C with constant mixing. Unbound protein was removed by washing the beads with coupling buffer and the remaining reactive groups were hydrolyzed with 0.1 M Tris-HCl, pH 8.0, overnight at 4°C with constant mixing. Beads were then washed by three cycles of alternating pH buffers (0.05 M Tris, pH 8.0, 0.5 M NaCl; and 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl) and finally equilibrated with extraction buffer (50 mM Tris-HCl, pH 7.4, 50 mM octyl-β-D-glucopyranoside, 15 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM PMSF, and 1 mM NEM). ^{35}S-CSPG were first precleared with mock beads by incubating overnight at 4°C before incubating with the protein-Sepharose 4B beads with constant mixing overnight at 4°C. The ^{35}S-CSPG/protein-Sepharose 4B slurries were then packed into columns, washed extensively with extraction buffer, and eluted with a linear salt gradient. Peak elution fractions were then pooled, dialyzed, and lyophilized. ^{35}S-CSPG that bound type IV collagen affinity columns were then resuspended and divided for the following experiments. ^{35}S-CSPG were labeled with 2.5 mCi ^{125}I (ICN Biomedicals) on iodobeads (Pierce Chemical, Rockford, IL) in 0.05 M Tris, pH 7.0, 0.5% CHAPS (Faassen *et al.*, 1992b) to visualize the core proteins in SDS-PAGE. ^{125}I-CSPG were applied onto DEAE-Sephacel (Sigma Chemical), washed with DEAE buffer (see above) to remove any unbound ^{125}I, eluted with 1.0 M NaCl-DEAE buffer, and dialyzed extensively in deionized water containing 1 mM PMSF. Samples were then dissolved in chondroitinase ABC (cABC) buffer (0.1 M Tris, 0.03 M sodium acetate, 0.01 M EDTA, 0.01 M NEM, 5 mM PMSF, pH 8.0) and digested with cABC (protease-free, Seikagaku America, Rockville, MD) at 0.1 U/ml. Samples were analyzed by nonreducing 7.5% SDS-PAGE with 5% stacking gels, dried, and autoradiographed onto Fuji RX film (Fuji Photo Film, Ashigara, Japan). To evaluate the ability of ^{35}S-GAG to bind type IV collagen and LM affinity columns, ^{35}S-GAG were released from PG core proteins by alkaline borohydride reduction, neutralized, and desalted on Sephadex G-50 (Sigma Chemical) columns, as previously described (Oegema *et al.*, 1979). ^{35}S-GAG were then precleared with mock beads by shaking overnight at 4°C before incubating with the protein-Sepharose 4B beads with constant mixing overnight at 4°C. ^{35}S-GAG/protein-Sepharose 4B slurries were then packed into columns, washed with extraction buffer, and batch eluted with 1 M NaCl extraction buffer.

Statistical Analyses

The level of statistical significance between control and experimental values within each experiment was calculated by the Student's two-tailed *t* test.

RESULTS

Cell Surface CSPG and α2β1 Integrin Mediate Human Melanoma Cell Invasion of Reconstituted BM

The invasion of cells through a reconstituted BM matrix in vitro has previously been shown to correlate with the ability to form metastatic tumors in vivo (Albini *et al.*, 1987). To evaluate the potential role of cell surface CSPG in mediating the invasive behavior of human melanoma cells, in vitro invasion through a reconstituted BM was quantitated following pretreatment of human melanoma cells with β-D-xyloside. Two different human melanoma cell lines were selected based upon their cell surface CSPG expression (Table 1), SK-MEL-2 (CD44 and MPG/NG2 positive) and M14#5 (CD44 positive and MPG/NG2 negative). Invasion of both cell lines was inhibited by >75% in the presence of β-D-xyloside and was not affected by the inactive analogue α-D-xyloside (Figure 1, A and B). Inhibition of melanoma cell invasion by β-D-xyloside treatment was not due to a cytotoxic effect of the pharmacological reagent based upon results of trypan blue exclusion (viability >95%), and the effects of β-D-xyloside treatment are reversible.

Previous studies have demonstrated a role for various integrins in the in vitro invasion of tumor cells. To confirm that integrins are also involved in promoting human melanoma cell invasion of BM, we then examined the abilities of several anti-integrin subunit mAbs to inhibit human melanoma cell invasion of reconstituted BM. Anti-integrin subunit mAbs tested included α2, α3, α5, and β1. As expected, the anti-integrin subunit mAbs against α2, α3, and β1 all significantly

Table 1. Flow cytometric analysis of human melanoma cell surface receptors

Antigen	mAb	SK-MEL-2 cells	M14#5 cells
[a]	FITC-2°	529[b]	566
[a]	n. mouse IgG	550	574
CD44	HAR 1968	983	984
MPG/NG2	9.2.27	854	570
β1	P5D2	850	758
α2	P1E6	756	691
α5	P1D6	612	608

Flow cytometric analysis of SK-MEL-2 and M14#5 human melanoma cell surface receptors indicates similar levels of expression of cell surface receptors with the exception of MPG/NG2) (the M14#5 cell line is negative for MPG/NG2). Cells were prepared for flow cytometric analysis as described in MATERIALS AND METHODS. mAbs used to evaluate cell surface expression of CSPG and integrin subunits included (anti-CD44, HAR 1968; anti-MPG/NG2, 9.2.27; and anti-β1, P5D2; -α2, P1E6; and -α5, P1D6).

[a] Negative controls.

[b] Values are expressed in terms of MFU (mean fluorescence units).

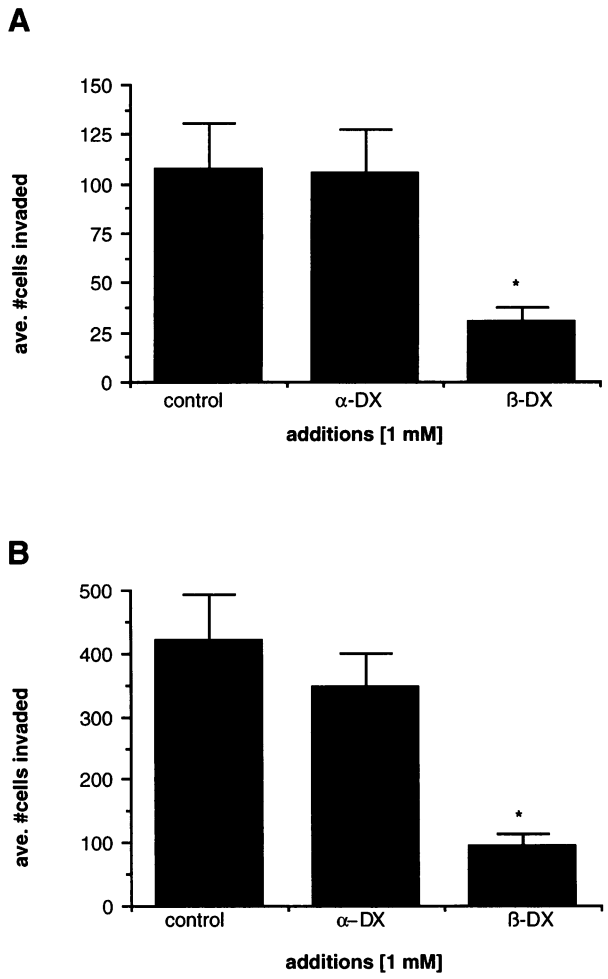

Figure 1. Human melanoma cell invasion of reconstituted BM is inhibited following pretreatment with β-D-xyloside. SK-MEL-2 (A) and M14#5 (B) melanoma cells were preincubated with or without 1 mM β-D-xyloside or α-D-xyloside for 24 h before assessing invasion in an overnight invasion assay (see MATERIALS AND METHODS). Invasion was specifically inhibited in the presence of β-D-xyloside, not by α-D-xyloside. This inhibition was statistically significant (* values represent $p < 0.05$; compared with control cells) when analyzed using the Student's two-tailed *t* test. Data represent the means of triplicate determinations ± SEM.

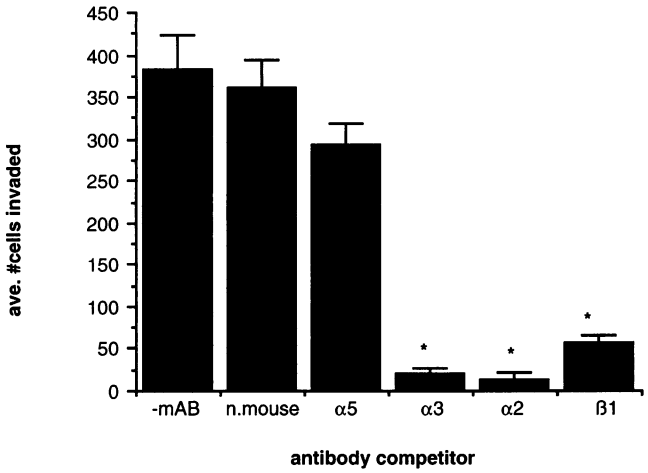

Figure 2. Human melanoma cell invasion of reconstituted BM is inhibited with anti-integrin subunit mAbs. SK-MEL-2 cells were preincubated with or without normal mouse IgG, anti-α2, -α3, -α5, and -β1 mAbs to evaluate possible inhibitory effects on invasion of human melanoma cells through reconstituted BM (see MATERIALS AND METHODS). Cells were preincubated in the presence or absence of mAbs for 20 min before addition to the invasion chambers. Anti-α2, -α3, and -β1 mAbs significantly inhibited melanoma cell invasion (* values represent $p < 0.05$; compared with control cells), in contrast, anti-α5 mAb and normal mouse IgG were unable to inhibit invasion. Data represent the means of triplicate determinations ± SEM.

inhibited invasion (Figure 2; $p < 0.05$). The anti-α5 mAb (an integrin subunit important for cellular recognition of fibronectin) was used as a negative control and was unable to significantly inhibit melanoma cell invasion (Figure 2). Collectively, the data show that in addition to cell surface CSPG, the type IV collagen integrins α2β1 and α3β1 are also involved in mediating human melanoma cell invasion of reconstituted BM.

Human Melanoma Cells May Express Two Different Cell Surface CSPG

Human melanoma cells can potentially express at least two different cell surface CSPG, CD44 and MPG/NG2 (Bumol and Reisfeld, 1982; Harper and Reisfeld, 1983; see reviews by Faassen *et al.*, 1992a; Lesley *et al.*, 1993). Flow cytometric analyses were performed with SK-MEL-2 and M14#5 human melanoma cells to evaluate their expression of these two CSPG core proteins in addition to several integrin subunits. The mean fluorescence units of various cell surface receptors expressed by these two melanoma cell lines are represented in Table 1. These results indicate the presence of high levels of CD44 on both SK-MEL-2 and M14#5 cells and high levels of MPG/NG2 only on SK-MEL-2 cells. To determine whether β-D-xyloside treatment had any inhibitory effects on the expression of various cell surface receptors, flow cytometry was also performed with β-D-xyloside– and α-D-xyloside–treated cells. β-D-xyloside pretreatment did not alter SK-MEL-2 or M14#5 human melanoma cell expression of cell surface CD44 and MPG/NG2 or several integrin subunits, α2, α3, α5, and β1 (our unpublished observations).

Human Melanoma Cell CD44/CSPG Is Important for Mediating Invasion of Reconstituted BM

To determine the involvement of CD44/CSPG and/or MPG/NG2 in the invasion of human melanoma cells through reconstituted BM, anti-CD44 (Hermes-3, Jalkanen *et al.*, 1987) and anti-MPG/NG2 mAb (9.2.27, Bumol and Reisfeld, 1982) were evaluated for any

possible inhibitory effect(s) in an overnight invasion assay. SK-MEL-2 (Figure 3A) and M14#5 (Figure 3B) human melanoma cells were preincubated for 20 min with the mAbs before addition to invasion chambers. Human melanoma cell invasion was inhibited by 60–70% upon preincubation with anti-CD44 mAb (Figure 3, A and B). Although in this experiment there was a slight inhibitory effect observed upon preincubation of SK-MEL-2 cells with anti-MPG/NG2 mAb (Figure 3A), it was not statistically significant ($p < 0.09$). Furthermore, MPG/NG2-deficient human melanoma cells, M14#5 cells (Figure 3B), were also capable of invading reconstituted BM and this invasion was inhibited by anti-CD44 mAb and not anti-MPG/NG2 mAb. These results indicate the inhibitory effects of anti-CD44 mAb are specific in nature and that CD44/CSPG is necessary for promotion of human melanoma cell invasion through reconstituted BM.

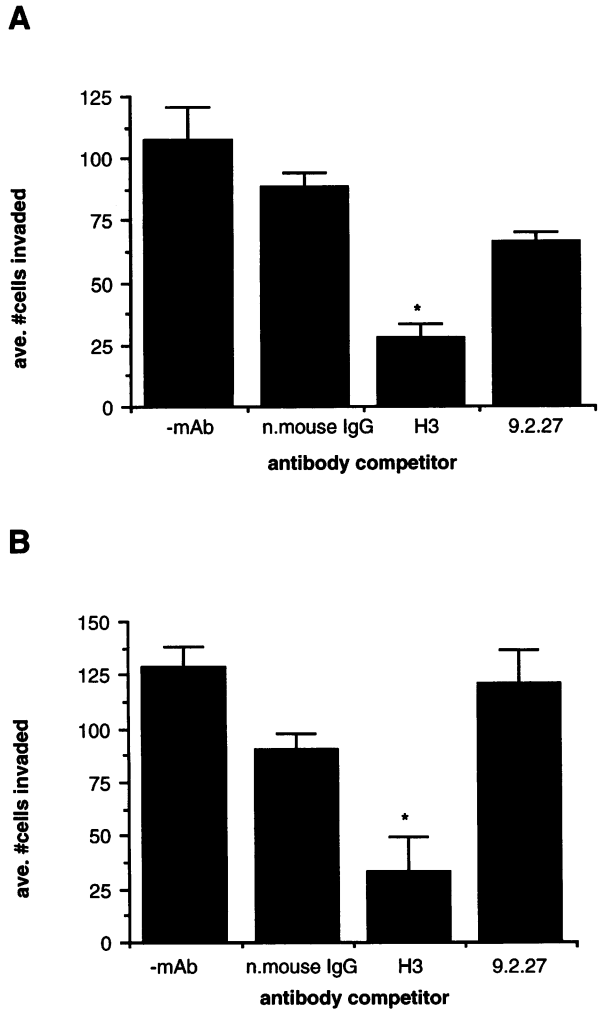

Figure 3. Human melanoma cell invasion of reconstituted BM is inhibited with anti-CD44 mAb. SK-MEL-2 (A) and M14#5 (B) cells were preincubated with or without anti-CD44 (H3: Hermes-3) and anti-MPG/NG2 (9.2.27) mAbs to evaluate possible inhibitory effects on invasion of human melanoma cells through reconstituted BM (see MATERIALS AND METHODS). Cells were preincubated with or without mAbs or normal mouse IgG for 20 min before adding to the invasion chambers. Data represent the means of triplicate determinations ± SEM (* values represent $p < 0.05$; compared with control cells).

Cell Surface CD44/CSPG and α2β1 Integrin Are Involved in Human Melanoma Cell Migration on Type IV Collagen, but not on LM

Previous studies have identified that CD44/CSPG expressed by mouse melanoma cells is important for migration on, and invasion of, type I collagen gels (Faassen *et al.*, 1992b, 1993). To further investigate the interaction of cell surface CD44/CSPG with BM, the effect of β-D-xyloside treatment on human melanoma cells was evaluated to determine whether CSPG were necessary for promotion of adhesion and/or migration on the isolated BM components, type IV collagen, and LM. Following pretreatment with β-D-xyloside, we quantitated human melanoma cell migration and adhesion on purified type IV collagen and LM. Human melanoma cell migration on type IV collagen was significantly inhibited following pretreatment with 1 mM β-D-xyloside (Figure 4A; $p < 0.001$). In contrast, migration on LM was unaffected by β-D-xyloside treatment. The inhibition on type IV collagen was specific for β-D-xyloside treatment as seen by the inability of α-D-xyloside treatment to inhibit melanoma cell migration on either type IV collagen or LM. In contrast, β-D-xyloside was unable to inhibit the adhesion response of human melanoma cells on either BM component (Figure 4B). Treatment of human melanoma cells with cABC produced similar results (our unpublished observations). Collectively, the data suggest that human melanoma cell surface CSPG specifically function to mediate migration on type IV collagen rather than acting as primary cell adhesion receptors for this BM component. Additionally, neither melanoma cell adhesion nor migration on LM is dependent upon the expression of cell surface CSPG.

To further evaluate the role of CSPG core protein (i.e., CD44 and/or MPG/NG2) in human melanoma cell interaction with the BM components, type IV collagen, and LM, adhesion and migration assays were performed in the presence of anti-CD44 and anti-MPG/NG2 mAbs. SK-MEL-2 human melanoma cell migration on type IV collagen was inhibited by 55% in the presence of anti-CD44 mAb and was unaffected by preincubation with anti-MPG/NG2 mAb or normal mouse IgG (Figure 5A). In contrast, human melanoma cell migration on LM was not inhibited by either Hermes-3, 9.2.27, or normal mouse IgG (Figure 5A). Although melanoma cell migration on type IV collagen was inhibited by preincubation with anti-CD44 mAb, adhesion to type IV collagen and LM (Figure 5B)

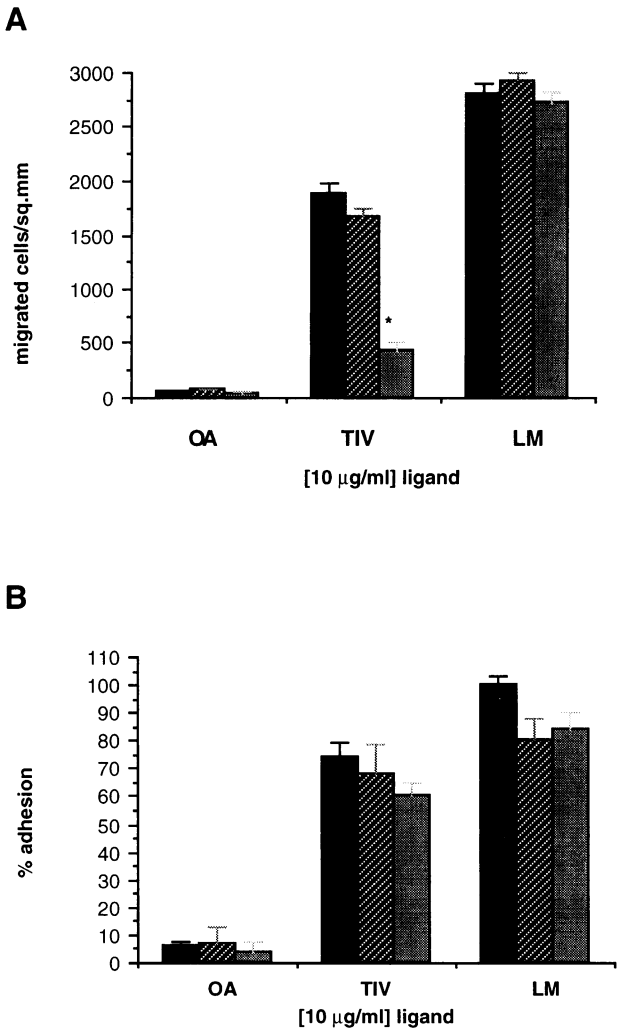

Figure 4. Human melanoma cell migration on type IV collagen (TIV) is inhibited following pretreatment with β-D-xyloside. SK-MEL-2 cells were preincubated in 1 mM β-D-xyloside (stippled bars) or its inactive analogue, α-D-xyloside (cross-hatched bars) for 24 h (untreated/control cells, solid bars). (A) The ability of cells to migrate in response to 10 μg/ml of TIV and LM was assessed in a modified Boyden chamber. β-D-xyloside was able to specifically inhibit melanoma cell migration on TIV by 60% (* value represents $p < 0.05$; compared with control cells), whereas α-D-xyloside was unable to inhibit melanoma cell migration on TIV or LM. (B) Cells were radiolabeled with [^{3}H]thymidine for 18 h before adhering on 10 μg/ml TIV- or LM-coated substrata. Melanoma cell adhesion was not inhibited by pretreatment with β-D-xyloside on either TIV or LM. OA represents ovalbumin coupled to itself and is included as a reference for background levels of migration and adhesion. Data represent the mean percentage of triplicate determinations ± SEM.

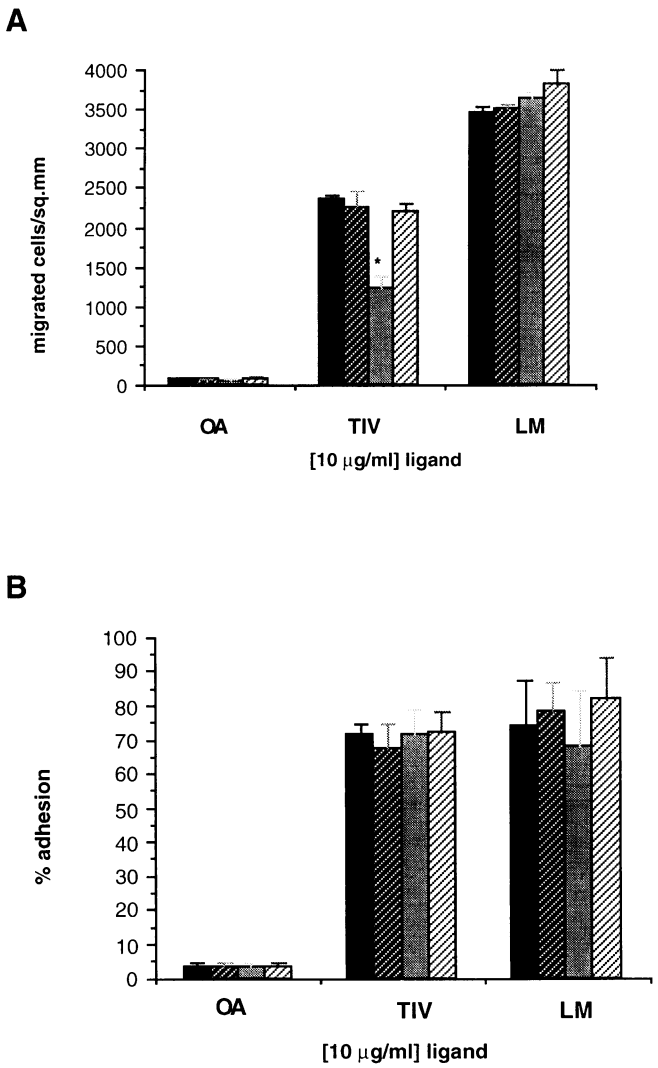

Figure 5. Human melanoma cell migration on TIV is specifically inhibited by anti-CD44 mAb. SK-MEL-2 cells were preincubated with either a 1:200 dilution of anti-CD44 mAb (gray stippled bars), 5 μg/ml of anti-MPG/NG2 mAb (gray hatched bars), or 5 μg/ml normal mouse IgG (black hatched bars) for 20 min before performing migration and adhesion assays (see MATERIALS AND METHODS). The solid black bars represent untreated/control cells. (A) The ability of cells to migrate in response to 10 μg/ml of TIV and LM was assessed in a modified Boyden chamber. Anti-CD44 mAb was able to specifically inhibit melanoma cell migration on TIV by approximately 50% (* value represents $p < 0.05$; compared with control cells). (B) The ability of cells to adhere to 10 μg/ml TIV and LM was not affected by the presence of anti-CD44 or anti-MPG/NG2 mAbs. Data represent the mean percentage of triplicate determinations ± SEM.

was not inhibited by any of the mAb preincubations, indicating that CD44 and MPG/NG2 are not serving as primary cell adhesion receptors for these BM ligands.

We then evaluated the ability of purified human melanoma cell surface CSPG to bind directly to both type IV collagen and LM. Cell surface PG expressed by human melanoma cells were isolated and partially characterized following previously described extraction procedures (Faassen *et al.*, 1992b, 1993). Detergent-extracted $^{35}SO_4$-labeled melanoma cell cultures were purified by HPLC-DEAE column chromatogra-

phy (Figure 6). Three peaks were recovered following elution with a linear salt gradient at 0.14 M NaCl, 0.34 M NaCl, and 0.42 M NaCl. The first two peaks (eluting at 0.14 M NaCl and 0.34 M NaCl) contained ^{35}S-glycoproteins and ^{35}S-heparan sulfate PG, respectively, and were not further characterized in these studies. The peak eluting at 0.42 M NaCl (~90% of the total eluted ^{35}S-labeled material) contained >95% CSPG as determined by sensitivity to cABC and resistance to nitrous acid following alkaline borohydride treatment to release the ^{35}S-labeled GAG from the protein cores. HPLC-purified CSPG or purified CS were then applied to affinity columns containing type IV collagen or LM and eluted with a linear salt gradient. Both the HPLC-purified CSPG (eluted at moderate salt conditions, 0.25–0.3 M NaCl) and purified CS bound to type IV collagen (Figure 7, A and B, respectively). In contrast, purified CSPG or CS does not bind to LM affinity columns, even under the very permissive binding conditions used in these studies (our unpublished observations). Human melanoma CSPG that bound type IV collagen affinity columns were then iodinated and subjected to SDS-PAGE analysis to visualize the core proteins. SK-MEL-2 and M14#5 CSPG core proteins were detected following cABC digestion (Figure 8). The M_r of the major band in the cABC-digested lanes, 2 and 4, is approximately 100 K, which is consistent with the M_r of CD44/CSPG core protein immunoprecipitated from whole cell lysates (our unpublished observations). Additionally, a faint band appears upon cABC digestion in lane 2 of the SK-MEL-2 CSPG with a M_r of 250 K (consistent with the M_r of MPG/NG2).

In addition to the role for human melanoma cell surface CD44/CSPG in the promotion of melanoma cell migration on type IV collagen and invasion of BM, our study has also confirmed a role for type IV collagen integrins in mediating melanoma cell invasion through a reconstituted BM. Previous studies have demonstrated a role for $\alpha 2\beta 1$ integrin in the in vitro invasion of tumor cells and for melanoma cell migration and adhesion on type IV collagen. To evaluate

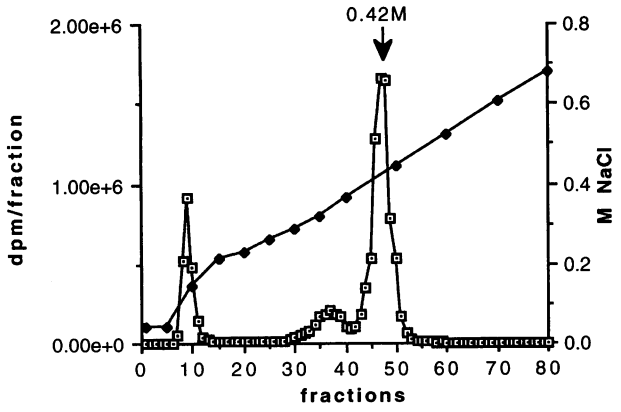

Figure 6. Human melanoma cell surface CSPG purified by HPLC-DEAE chromatography. Detergent-extracted SK-MEL-2 ^{35}S-PG were applied to an HPLC-DEAE column in DEAE buffer and eluted with an NaCl gradient (see MATERIALS AND METHODS). Radioactivity was monitored for each 1 ml fraction (open squares) and the salt gradient was monitored by conductivity measurements (closed diamonds).

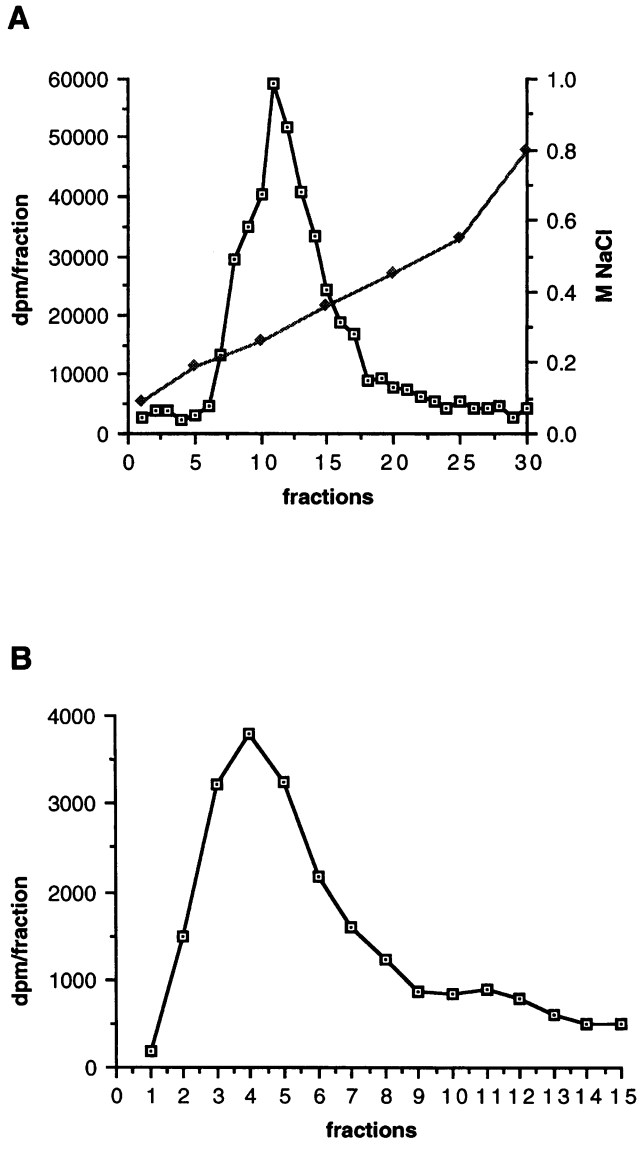

Figure 7. Purified human melanoma cell surface CSPG and CS bind type IV collagen affinity columns. HPLC-purified ^{35}S-CSPG that had been precleared with a mock column (see MATERIALS AND METHODS) were incubated with a type IV collagen affinity column and eluted with a linear salt gradient (A). To evaluate ^{35}S-GAG binding to a type IV collagen affinity column, alkaline borohydride-released ^{35}S-GAG were also precleared with a mock column, applied to a type IV collagen affinity column, and batch-eluted with 1 M NaCl extraction buffer (B) (see MATERIALS AND METHODS).

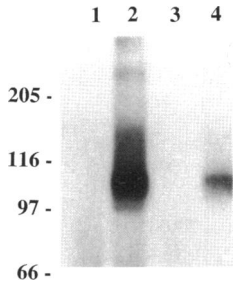

Figure 8. SDS-PAGE of human melanoma cell surface CSPG that bind type IV collagen affinity columns. ^{35}S-CSPG (lanes 1 and 2, SK-MEL-2; lanes 3 and 4, M14#5) that bound a type IV collagen affinity column were labeled with ^{125}I to visualize the core proteins in 7.5% SDS-PAGE (see MATERIALS AND METHODS). Lanes 1 and 3 represent undigested, lanes 2 and 4 represent cABC-digested ^{125}I-CSPG. Upon cABC-digestion, bands of approximately 100 K appear in the gel (nonreduced; M_r does not shift upon reduction, our unpublished observations). Undigested ^{125}I-CSPG is not present in the photograph due to its location in the stacking gel.

what melanoma cell surface receptor(s) may be functioning as an adhesion receptor for type IV collagen and/or LM, we then examined the ability of several anti-integrin subunit mAbs to inhibit human melanoma cell adhesion to type IV collagen and LM. Human melanoma cell adhesion and migration on type IV collagen and LM were both inhibited by the anti-β1 integrin subunit mAb (Figure 9, A and B). The anti-α2 integrin subunit mAb, however, was only able to inhibit human melanoma cell adhesion and migration on type IV collagen, not on LM, indicating that melanoma cells use $\alpha2\beta1$ integrin to adhere and migrate on type IV collagen. The anti-α5 subunit mAb (used as a negative control) was unable to inhibit adhesion or migration on either type IV collagen or LM (Figure 9, A and B). Furthermore, the inhibitory effects of anti-α2 or -β1 subunit mAbs and β-D-xyloside treatment on melanoma cell adhesion and migration on type IV collagen were not additive (our unpublished observations). In addition, an anti-α3 mAb was ineffective at inhibiting melanoma cell adhesion and migration to either type IV collagen or LM (our unpublished observations), suggesting that these cells do not use $\alpha3\beta1$ integrin for recognition of these BM components. Collectively, these results indicate that human melanoma cell recognition of type IV collagen is an important event in the invasion of reconstituted BM in vitro and suggest that CD44/CSPG may act with $\alpha2\beta1$ in promoting human melanoma cell adhesion, migration, and invasion in vivo via a type IV collagen-dependent mechanism.

DISCUSSION

Tumor cell invasion through BM is a complex process and is dependent, in part, upon cell adhesion and migration. Although many previous studies have emphasized an importance of LM in mediating melanoma invasion of BM, the current studies demonstrate an importance for tumor cell adhesion and migration on type IV collagen in this process. Specifically, interference with CD44/CSPG expression by using β-D-xyloside or anti-CD44 mAbs could inhibit both invasion of reconstituted BM and migration on type IV collagen. However, melanoma cell adhesion to type IV collagen was essentially unaffected by these treatments, consistent with previous studies in which interference with CD44/CSPG expression in mouse melanoma cells inhibited migration, but not adhesion, of these cells on type I collagen (Faassen *et al.*, 1992b, 1993). Adhesion of melanoma cells to type IV collagen was instead found to depend primarily upon the action of $\alpha2\beta1$ integrin, and anti-α2 or anti-β1 integrin mAbs could also inhibit melanoma cell invasion of reconstituted BM. Collectively, our data suggest a model in which human melanoma cell CD44/CSPG and $\alpha2\beta1$ integrin may both mediate melanoma cell interaction with type IV collagen and facilitate invasion of BM in vivo.

CD44 comprises a group of cell surface glycoproteins that bind to various ECM components and modulate cell migration, lymphocyte homing, and hematopoiesis, among other things (see review by Lesley *et al.*, 1993). Various molecular isoforms are produced by alternative splicing of transcripts from a single gene, and these isoforms can be post-translationally modified in several ways, contributing to a high degree of structural and functional heterogeneity. Several studies have implicated an association between the expression of different splice variants of CD44 (i.e., v6 isoforms containing exon 10) and the metastatic potential of certain tumors (Gunthert *et al.*, 1991; Matsumura and Tarin, 1992; Rudy *et al.*, 1993). Other studies have demonstrated a general correlation between CD44 levels and tumor cell migration and invasion on various ligands (Birch *et al.*, 1991; Honn and Tang, 1992; Thomas *et al.*, 1993). In addition, anti-sense inhibition of CD44 expression in glioma cells inhibits their in vitro invasion (Merzak *et al.*, 1994). Collectively, these results demonstrate an important role for the CD44 core protein in modulating the invasion and metastasis of multiple types of tumors, although the mechanism(s) of action of CD44 is not well understood.

The addition of CS to the CD44 core protein is fundamentally important for mediating the binding of CD44/CSPG to type IV collagen. Alkaline borohydride-released CS bound directly to a type IV collagen affinity column, whereas the CD44 core protein that lacked CS failed to bind this column (our unpublished observations). The present study demonstrates a role for CD44/CSPG in mediating human melanoma cell migration on type IV collagen and invasion of a more complex environment (i.e., reconstituted BM). Because removal of the CS by β-D-xyloside or cABC (our unpublished observations) can inhibit melanoma cell migration on type IV collagen, it would seem that an important role for the CS is to mediate the binding of CD44/CSPG to type IV collagen. Importantly, the interaction of CD44/CSPG with type IV collagen is rel-

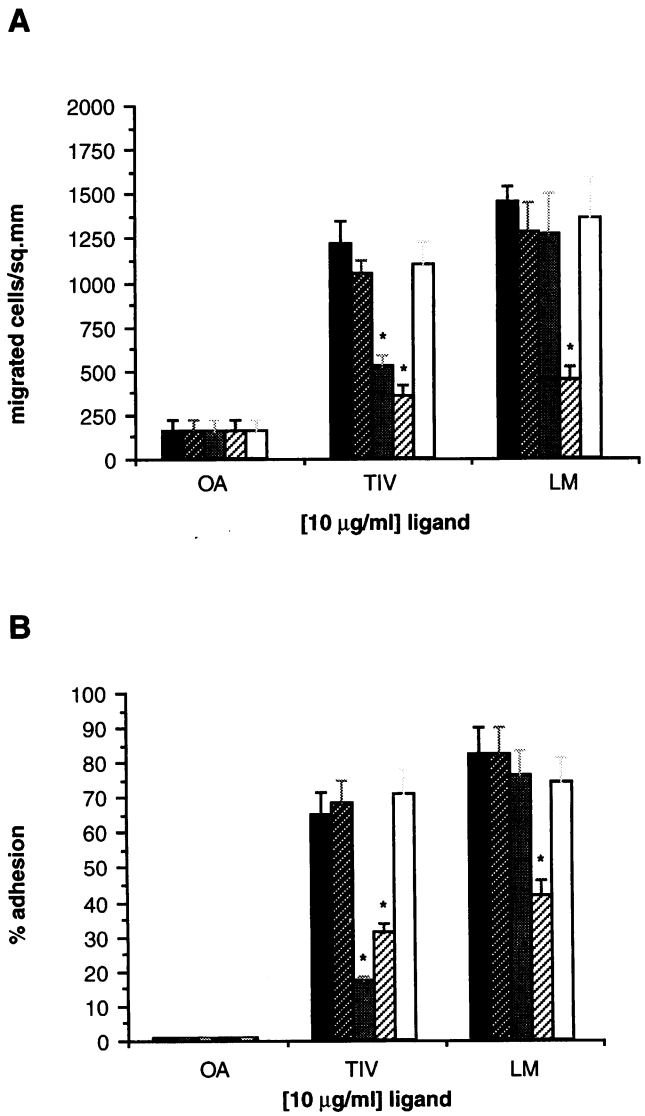

Figure 9. Human melanoma cell migration and adhesion on type IV collagen are both inhibited with anti-integrin subunit mAbs. SK-MEL-2 cells were preincubated with either a 1:200 dilution of ascites mAb or 5 μg/ml purified IgG (normal mouse, anti-α2, and -β1) for 20 min before performing migration and adhesion assays (see MATERIALS AND METHODS). Black bars represent untreated/control cells, black hatched bars represent normal mouse IgG treated cells, gray stippled bars represent α2 treated cells, gray hatched bars represent β1 treated cells, and white bars represent α5-treated cells. (A) Cell migration in response to 10 μg/ml of TIV and LM was assessed in a Boyden chamber. Anti-α2 mAb was able to specifically inhibit melanoma cell migration on TIV by approximately 50%, whereas anti-β1 mAb was able to inhibit melanoma cell migration on both TIV and LM by >80% (* values represent $p < 0.05$; compared with control cells). (B) The ability of cells to adhere to 10 μg/ml TIV was inhibited by both anti-α2 and -β1 mAbs (>50% and >80%, respectively), whereas the ability of cells to adhere to 10 μg/ml LM was inhibited only by the anti-β1 mAb (* values represent $p < 0.05$; compared with control cells). Data represent the mean percentage of triplicate determinations ± SEM.

atively specific, because this PG fails to bind LM and removal of CS on melanoma cells has no detectable effect on LM-mediated cell adhesion and migration. Whether there are specific sulfation patterns associated with melanoma-associated CS that might contribute to ligand binding properties of the CD44/CSPG to type IV collagen remains an open, and potentially interesting question.

In addition to CD44, human melanoma cells can also express another cell surface CSPG core protein, termed MPG/NG2 (Bumol and Reisfeld, 1982; Harper and Reisfeld, 1983; Harper *et al.*, 1984; Spiro *et al.*, 1991). MPG/NG2 has previously been shown to bind to type VI collagen, and anti-MPG/NG2 antibodies have been reported to inhibit melanoma cell adhesion and/or spreading on isolated ECM proteins (Harper *et al.*, 1984; Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993) and cultured endothelial cells (De Vries *et al.*, 1986). MPG/NG2 has also been implicated in modulating the invasion of melanoma cells in vitro (Chattopadhyay *et al.*, 1991, 1992). In those studies, polyclonal antibodies raised against an MPG/NG2-specific anti-idiotope mAb could recognize MPG/NG2-positive melanoma cells, immunoprecipitate MPG/NG2, and could also inhibit melanoma cell invasion in vitro. Although the above studies collectively demonstrate an important role for MPG/NG2 in modulating melanoma cell adhesion or invasion, our current studies suggest that MPG/NG2 expression is not required for melanoma cell migration on type IV collagen or invasion through BM. Clearly, additional work is necessary to further clarify the role of MPG/NG2 in mediating human melanoma cell recognition of the ECM or other cells.

Although inhibiting CD44/CSPG expression and/or function clearly inhibited melanoma cell migration on type IV collagen, it had no effect on modulating cell adhesion to this ligand. Therefore, we performed additional studies to evaluate the potential nature of the melanoma integrins that are important for cellular recognition of type IV collagen. These results demonstrated that both cell types used in this study relied mainly on α2β1 integrin for adhering to type IV collagen. Although the anti-α3 integrin mAb was effective at inhibiting invasion of BM, it was ineffective at inhibiting melanoma cell adhesion or migration on type IV collagen or LM (our unpublished observations). Thus, our results are in contrast to several other studies that have demonstrated that α2β1 integrin can interact with both type IV collagen and LM (Elices and Hemler, 1989; Etoh *et al.*, 1992), or that α3β1 integrin can interact with both proteins (Yoshinaga *et al.*, 1993; Melchiori *et al.*, 1995). However, there are additional studies that demonstrate that α2β1 integrin is primarily a type IV collagen receptor for melanoma cells (Kramer and Marks, 1989; Mortarini *et al.*, 1991) and that α3β1 integrin has no role in mediating melanoma

cell migration on type IV collagen (Etoh *et al.*, 1993). Although the exact reasons for these discrepancies are not understood, they may be related to the expression of variant integrin isoforms with different ligand binding specificities (Altruda *et al.*, 1990; Tamura *et al.*, 1991). Alternatively, the differences may be due to cell type–specific variations in the activation state of the integrins (Danen *et al.*, 1993), which may reflect the involvement of different signaling mechanisms.

Collectively, our data present a model system in which $\alpha2\beta1$ integrin and CD44/CSPG are both required for motile and invasive activity of human melanoma cells. In this model, $\alpha2\beta1$ integrin is involved in melanoma cell adhesion and migration on type IV collagen, whereas CD44/CSPG does not appear to be required for melanoma cell adhesion but is necessary for post-adhesion events that are required for migration on type IV collagen. Although $\alpha3\beta1$ integrin is also apparently involved in melanoma cell invasion of BM, it is unclear at this point what specific role it plays in this model system in promoting invasion. Using these cells, an anti-$\alpha3$ integrin mAb was not able to inhibit melanoma cell adhesion or migration on type IV collagen or LM, despite the fact that it effectively inhibits invasion. One possibility is that $\alpha3\beta1$ integrin could be linked with the expression and/or release of specific BM proteases as has been described in other systems (Larjava *et al.*, 1993; Seftor *et al.*, 1993; Seltzer *et al.*, 1994; Riikonen *et al.*, 1995).

Our results suggest that adhesion and migration on type IV collagen may involve some sort of specific cooperative mechanism that requires both CD44/CSPG and $\alpha2\beta1$ integrin. It is apparently important that both receptors must bind type IV collagen because the migration of β-D-xyloside–treated melanoma cells is significantly impaired, despite the fact that $\alpha2\beta1$ integrin can still support cell adhesion to this protein. These results suggest that both CD44/CSPG and $\alpha2\beta1$ integrin have to be in close proximity on the plasma membrane, or engaged by type IV collagen in a specific temporal pattern, for this interaction to occur. In support of this model, we have identified a type IV collagen-derived synthetic peptide, L-IV-H1 [$\alpha1$(IV)1263–1277], that promotes melanoma cell adhesion and migration. This peptide binds both CD44/CSPG and $\alpha2\beta1$ integrin independently, and the binding of both receptors is required for melanoma cell adhesion and migration (Knutson *et al.*, unpublished data).

Although the nature of the interaction of these two receptors is not known, one possibility is that CD44/CSPG could act to stabilize the leading edge of migrating cells, similar to what we have previously observed in studying a role for this receptor in migration on type I collagen (Faassen *et al.*, 1993). Such a model is consistent with our observation that inhibiting both receptors does not have an additive effect on the adhesion of cells on type IV collagen (our unpublished observations). Additional studies are now focused on evaluating the possible mechanism(s) of CD44/CSPG and $\alpha2\beta1$ integrin cooperation in promoting melanoma cell adhesion and migration. Such mechanisms could include interactions between the ectodomain of the integrin and CD44/CSPG, whereby both receptors may influence either ligand binding and/or clustering shown to be important for signal generation by both types of receptors (Miyamoto *et al.*, 1995; Perschl *et al.*, 1995). Alternatively, such mechanisms may also involve signaling through changes in the cytoskeleton or other signaling pathways that have been shown to be modified by CD44 (Jacobson *et al.*, 1984; Tarone *et al.*, 1984; Lacy and Underhill, 1987; Carter and Wayner, 1988; Kalomiris and Bourguignon, 1988; Geppert and Lipsky, 1991; Bourguignon *et al.*, 1992) or $\alpha2\beta1$ integrin (Kapron-Bras *et al.*, 1993; Leavesley *et al.*, 1993; Sanchez-Mateos *et al.*, 1993).

Finally, our results raise an intriguing question with respect to mechanism(s) of tumor cell invasion of intact BM and suggest that interaction of melanoma cells with both type IV collagen and LM are each required, but not sufficient, to promote melanoma cell invasion. Although the reasons for this are unclear, one possibility is that there are several distinct, yet equally required, signaling pathways involved in tumor cell invasion invoked via different cell surface receptors (i.e., CD44/CSPG, type IV collagen- and/or LM-specific integrins). How and at what point these distinct pathways might converge is an important future area for investigation. Knowledge of the complex interplay between signaling mechanisms involved in melanoma cell recognition of type IV collagen and LM should enhance our understanding of the ways in which complex ECM structures modulate normal and transformed cell behavior, and should add new awareness to the detailed mechanisms involved in tumor cell invasion and metastasis.

ACKNOWLEDGMENTS

The authors thank Drs. Eugene Butcher, Ralph Reisfeld, and Leo Furcht for generously providing mAbs used in these studies. In addition, we thank Dr. Barbara Mueller for providing the M14#5 human melanoma cell line. This work was supported by National Institutes of Health grants KD-44494 and AR-01929, the Leukemia Task Force, and the American Cancer Society grant CB-101. This manuscript is part of a doctoral thesis by J.R.K. in partial fulfillment of the requirements of the University of Minnesota Graduate School.

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