A Cell Surface Chondroitin Sulfate Proteoglycan, Immunologically Related to CD44, Is Involved in Type I Collagen-mediated Melanoma Cell Motility and Invasion

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Abstract. The metastatic spread of tumor cells occurs through a complex series of events, one of which involves the adhesion of tumor cells to extracellular matrix (ECM) components. Multiple interactions between cell surface receptors of an adherent tumor cell and the surrounding ECM contribute to cell motility and invasion. The current studies evaluate the role of a cell surface chondroitin sulfate proteoglycan (CSPG) in the adhesion, motility, and invasive behavior of a highly metastatic mouse melanoma cell line (K1735 M4) on type I collagen matrices. By blocking mouse melanoma cell production of CSPG with p-nitrophenyl-ß-D-xylopyranoside (ß-D-xyloside), a compound that unouples chondroitin sulfate from CSPG core protein synthesis, we observed a corresponding decrease in melanoma cell motility on type I collagen and invasive behavior into type I collagen gels. Melanoma cell motility on type I collagen could also be inhibited by removing cell surface chondroitin sulfate with chondroitinase. In contrast, type I collagen-mediated melanoma cell adhesion and spreading were not affected by either ß-D-xyloside or chondroitinase treatments. These results suggest that mouse melanoma CSPG is not a primary cell adhesion receptor, but may play a role in melanoma cell motility and invasion at the level of cellular translocation. Furthermore, purified mouse melanoma cell surface CSPG was shown, by affinity chromatography and in solid phase binding assays, to bind to type I collagen and this interaction was shown to be mediated, at least in part, by chondroitin sulfate. Additionally we have determined that mouse melanoma CSPG is composed of a 110-kD core protein that is recognized by anti-CD44 antibodies on Western blots. Collectively, our data suggests that interactions between a cell surface CD44-related CSPG and type I collagen in the ECM may play an important role in mouse melanoma cell motility and invasion, and that the chondroitin sulfate portion of the proteoglycan seems to be a critical component in mediating this effect.

Tumor cell metastasis involves a complex series of interdependent events, one of which involves the adhesion of tumor cells to extracellular matrix (ECM) components (Liotta et al., 1983). Although traditionally considered as structural elements of the ECM, it is now appreciated that various collagen types promote the adhesion and migration of normal and transformed cells (Aumaillé and Timpl, 1986; Rubin et al., 1981; Dedhar et al., 1987; Herbst et al., 1988; Chelberg et al., 1989). Additionally, certain collagen types have been shown to promote directional motility of normal and transformed cells, thus, potentially contributing to the invasive process (Herbst et al., 1988, Chelberg et al., 1989). The motility of normal and transformed cells may be directed by an adhesive gradient of substratum-bound attractant, and this process is termed haptotaxis (McCarthy and Furcht, 1984).

Tumor cells adhere and move on ECM components via multiple cell surface receptors that interact with distinct domains on ECM proteins. In addition to the well known integrin receptor model for cell–ECM interactions (Buck and Horwitz, 1987, Hynes, 1987, Ruoslahti, 1988), substantial evidence suggests an important role for cell surface proteoglycans (PGs) in mediating cell adhesion to ECM components (Hook et al., 1984; Couchman and Hook, 1988; Ruoslahti, 1988; Wight, 1989). Both cell surface heparan sulfate proteoglycans (HSPG) and chondroitin sulfate proteoglycans (CSPG) have been implicated in modulating cell adhesion (Lark et al., 1985), but each has distinct effects on cell adhesion (Ruoslahti, 1988, Gallagher, 1989). While cell surface HSPG has been associated with the formation of tight cell adhesion contacts on ECM components (Lark et al., 1985;
Woods et al., 1986), cell surface CSPG has been implicated in weakening cell adhesion (Lark et al., 1985, Culp et al., 1978). Although the molecular mechanism by which CSPG disrupts cell adhesion is not understood, CSPG may facilitate cell detachment from the ECM, and thereby promote tumor cell motility and subsequent invasion.

To further evaluate the potential role of cell surface CSPG in tumor cell motility and invasion, we studied the effect of p-nitrophenyl β-D-xlyopyranoside (β-D-xlyoside), an agent that uncouples chondroitin sulfate addition to the protein core (Schwartz, 1977), on the invasion of melanoma cells into type I collagen gels. Others have shown that the inhibition of CSPG synthesis by β-D-xlyoside causes a dose-dependent inhibition of invasive morphogenetic events such as the branching of salivary glands (Thompson and Spooner, 1982) and ureteric buds (Klein et al., 1989). Our studies show that β-D-xlyoside inhibits the invasion of mouse melanoma cells into type I collagen gels, suggesting a role for cell surface CSPG in contributing to the invasive behavior of these cells. Similarly, haptoattactic motility of melanoma cells on type I collagen was inhibited by either β-D-xlyoside or chondroitinase pretreatment. However, type I collagen-mediated melanoma cell adhesion or spreading was not affected by these treatments. These data suggest that while melanoma CSPG may be important in cell motility at the level of translocation, it is apparently not a primary cell adhesion receptor. We have isolated and partially characterized a cell surface CSPG from mouse melanoma cells that interacts with type I collagen of the ECM and has properties consistent with localization as an integral membrane component. Furthermore, our data indicates that the 110-kD core protein of mouse melanoma CSPG is immunologically related to the CD44 antigen, implicated in several aspects of cell--cell and cell--ECM interactions (Gallatin et al., 1989; Haynes et al., 1989; Jalkanen et al., 1986; Miyake et al., 1990) and most recently implicated in mediating the metastatic behavior of certain carcinoma cell lines (Günthert et al., 1991). Thus, mouse melanoma cells express a cell surface CD44-related CSPG molecule that may play an important role in collagen-mediated melanoma cell motility and invasion.

Materials and Methods

Cell Culture

A highly metastatic clone (M4) of the K1735 mouse melanoma, generously provided by Dr. I. J. Fidler (M.D. Anderson Hospital Cancer Center, Houston, TX). This melanoma cell line was maintained in vitro culture in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (hyclone, Logan, UT). The medium was changed daily.

Invasion Gels

Gels composed of type I collagen were prepared under sterile conditions using a modified protocol as described by Shor (1980). Type I collagen (Collagen Corp., Palo Alto, CA; 3.3 mg/ml in 0.05M HCl) was mixed to a final concentration of 2.2 mg/ml in DMEM (Dulbecco's modified Eagle's medium; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. The number of cells in vitro passages was limited to eight in order to minimize phenotypic drift. There was no detectable change in the metastatic phenotype of these cells over this time, as measured by an experimental metastasis assay (McCarthy et al., 1986).

β-D-Xlyoside and Chondroitinase ABC Pretreatment

For the invasion assays, cells were seeded onto the gels as described above in the presence of 0, 0.05, 0.1, 0.5 or 1.0 mM p-nitrophenyl β-D-xlyopyranoside [β-D-xlyoside] (Sigma Chemical Co.) or p-nitrophenyl-α-D-xlyopyranoside [α-D-xlyoside] (Roche, Mannheim, Germany). For motility, adhesion and spreading assays, or before protoglycan extraction, cells were exposed to β-D-xlyoside for 48 h to inhibit CSPG synthesis by replacing the medium of cell cultures, at 50% confluence, with DMEM containing 2.5% heat-inactivated calf serum, with or without 1 mM α-D-xlyoside. α-D-xlyoside does not inhibit CSPG synthesis and was used as a control in invasion and motility assays. 1 mM α or β-D-xlyoside was also added to include the medium during the assays. β-D-xlyoside was also added to

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the 35S-sulfate labeling medium before proteoglycan extraction such that the cells were exposed to the drug for a total of 48 h (see below). Levels of protein synthesis were examined for cells pretreated (or not) with β-oxyloside. Briefly, cells grown in 96-well plates (Costar, Cambridge MA) were exposed (or not) to 1.0 mM β-oxyloside for 48 h and 5 μCi/ml 3H-leucine was added to each of triplicate wells for the final 6 h. Levels of 3H-leucine incorporation into cell-associated proteins were determined by harvesting cells with a Brandell cell harvester (Gaithersburg, MD), and the values were compared for cells exposed (or not) to β-oxyloside on a per cell basis. The latter value was obtained by counting cells/well (in triplicate), or determining relative DNA synthesis (0.2 μCi/μl 3H-thymidine labeled for 24 h). Radioactivity was quantitated by liquid scintillation spectroscopy (Beckman LS 3801).

For chondroitinase ABC treatment, cells were pretreated for 15 min with 0.1 U/ml chondroitinase ABC (Sigma Chemical Co.) before motility and adhesion assays, as described by Saunders and Bernfeld (1988). Chondroitinase ABC enzyme (0.1 U/ml) was also included in the cell medium during migration and adhesion assays. The absence of proteases in the chondroitinase ABC enzyme was verified by a lack of digestion of azocasein after a 60-min incubation with the enzyme at 37°C (Tomarelli et al., 1949). Chondroitinase ABC enzyme activity was verified for the assay conditions used, under conditions of Saito et al. (1968).

**Proteoglycan Extraction**

Prior to extraction, melanoma cell proteoglycans (PGs) were preferentially labeled with 35S-sulfate for 18 h by replacing the medium in 80% confluent cultures with RPMI 1640 containing 0.1 mM NaSO4, 0.36 mM glucose, and 50 μCi/ml Na35SO4 (Carrier free, Specific Activity 43 Ci/mg; ICN Biomedicals, Irvine, CA). An extraction protocol utilizing detergent-mediated cell lysis was followed to specifically enrich for intact plasma membrane-associated PG (Yanagashita et al., 1987). To remove peripheral, extrinsically associated PG, the medium of 35S-labeled cells was replaced with 30 ml DMEM containing 50 μg/ml heparin for a 30-min incubation at 18°C (Yanagashita and Hascall, 1984). The heparin extract was removed and 30 ml cellular extraction buffer (0.5 M NaCl, 0.1 M Tris, 5 mM MgCl2, 2 mM EDTA, 0.25 mM DTT. 1 mM PMSF, 1% Triton X-100, pH 7.2) was added to each roller bottle and incubated in a rolling apparatus at 37°C for 15 min (Carey and Todd, 1986). The cellular extracts, containing plasma membrane-associated and intracellular PG, were centrifuged at 1,500 rpm for 5 min to remove insoluble material. The remaining extracellular matrix–cytoskeletal-associated PG was solubilized in 4 M guanidine containing 50 mM NaAcetate, pH 5.8, 2% CHAPS, 0.05 M NaCl, 0.5 mM EDTA, 1 mg/ml benzamidine, 0.1 M 6-aminohexanoic acid, for a 30-min incubation at 25°C (Oegema et al., 1979). All extracts were dialyzed in small pore dialysis tubing (mol wt cut off 3,500) against successive changes of 0.5 M NaAcetate, pH 6.8, 0.1 M NaSulfate containing 10 mM EDTA, 0.1 mM PMSF, 10 mM 6-aminohexanoic acid (Oegema et al., 1979) until no radioactivity was observed in the dialysis buffer.

**Purification of Detergent-extracted CSPG**

Detergent-extracted 35S-PGs were dialyzed into DEAE buffer (0.15 M Tris, 6.0 M Urea, 0.1 M NaCl, 0.01 M EDTA, 0.01 M 6-aminohexanoic acid, 0.2% Triton X-100, 0.1 M Na2SO4, 0.1 M NaSulfate, pH 7.0), and purified by HPLC (Beckman Model 110 A) with a linear salt gradient 0.2% Triton X-100, 0.1 M Tris, 6.0 M Urea, 0.1 M NaCl, 0.01 M 6-aminohexanoic acid, 0.1 M Na2SO4, 0.36 M Glutamic acid, 0.1 M NaHCO3, 0.1 M Na2SO4, pH 7.2) and a flow rate of 3 ml/h (Klein et al., 1986). All 35S-PGs were washed with three column volumes of buffer and eluted with a salt gradient from 0.1 to 4.0 M NaCl. Detergent-extracted 35S-macromolecules resistant to the effects of nitrous acid and chondroitinase ABC digestion were considered as 35S-glycoproteins (Brown et al., 1981), and this population never exceeded 10% of any extract examined.

**Type I Collagen Affinity Chromatography and Binding Assays**

For affinity chromatography, type I collagen (Vitrogen) was covalently coupled to Reacti-Gel according to manufacturers instructions (Pierce Chemical Co.). Final concentration bound was 0.175 mg type I collagen/ml gel. Columns (30 ml bed volume) were equilibrated with 50 mM Tris, pH 6.8, 0.5% CHAPS, 0.05 M NaCl, 0.01 M 6-aminohexanoic acid, 0.1% Na2SO4, 1.0 mM PMSF, 0.002% azide. Detergent-extracted 35S-CSPG was applied to the columns, washed with three column volumes of buffer and eluted with a salt gradient from 0.05 to 1.0 M NaCl. To assure that the binding of CSPG was specific to type I collagen, 35S-CSPG was applied to duplicate columns prepared without type I collagen. Recovery of radioactivity from these columns was 90-95%.

Solid phase binding assays were performed with Immulon 1 plates as support (Skubitz et al., 1988). The substrata were prepared, as in the adhesion assays, by coating the surface of the wells with intact type I collagen (0.2 μg/well) or BSA (0.2 μg/well; fatty acid free; Sigma Chemical Co.). The plates were blocked for 1 h with 0.1 M carbonate buffer (Engvall and Perlman, 1972) containing 5 mg/ml BSA, then washed 5× with deionized water, and 35S-CSPG was added to the wells in binding buffer (50 mM Tris, pH 6.8, 50 mM NaCl, 5 mg/ml BSA). The plates were incubated for 2 h at 37°C, washed (x5) in binding buffer, and the bound 35S-CSPG was solubilized with 0.5 N NaOH containing 1% SDS.

**Ocyl Sepharose Chromatography**

Detergent-extracted 35S-CSPG, purified by ion exchange chromatography, was resuspended in Octyl Sepharose buffer (4.0 M Guanidine HCl, 20 mM Tris, pH 6.8). Samples were applied to a 5 ml Octyl Sepharose CL-4B (Sigma Chemical Co.) column at a flow rate of 0.5 ml/min. Hydrophobic CSPG were then eluted with a linear gradient of 0 to 0.5% Triton X-100 in Octyl Sepharose buffer (Yanagashita et al., 1987). 2 ml-fractions were analyzed for 35S-radioactivity and percent Triton X-100 by absorbance at 280 nm.

**Immunoblots**

For identification of CSPG core protein by immunoblot, detergent-extracted DEAE buffer containing 1.0 M NaCl, and dialyzed extensively in deionized water, 1 mM PMSF. Radioactivity per sample was quantitated by gamma ray scintillation (TM Analytic, Gamma Trac 1193). To visualize CSPG core protein by autoradiography, 351-CSPG was digested with chondroitinase ABC (Seikagaku America Inc., Rockville, MD) and analyzed by 6-15% SDS gel electrophoresis under nonreducing conditions (McCarthy et al., 1988a). Gels were dried under vacuum and used for autoradiography using Kodak XAR5.

**Characterization of Detergent-extracted CSPG**

The hydrodynamic properties of detergent-extracted 35S-PG and 35S-glycosaminoglycans (GAGs) were evaluated by gel filtration on 0.9 × 110 cm Sepharose CL-6B (Sigma Chemical Co.) columns. The columns were equilibrated and eluted with 0.5 M sodium acetate, pH 7.0, containing 0.2% CHAPS, at a flow rate of 3 ml/h (Klein et al., 1986). 35S-GAGs were released from PG protein cores by alkaline borohydride reduction, neutralized, and desalted on Sephadex G-50 (Sigma Chemical Co.) columns, as previously described (Oegema et al., 1979). 35S-GAGs were recovered from the column V, with <5% of alkali-released material included in the column. The heparan sulfate and chondroitin sulfate content of 35S-GAG samples were determined by sequential nitrous acid deaminative cleavage and chondroitinase ABC treatment, respectively, as previously described (Brown et al., 1981). The hydrodynamic properties of 35S-chondroitin/dermatan sulfate recovered after nitrous acid treatment were determined on Sepharose CL-6B columns. The C4:6 ratio of oligosaccharides generated by chondroitinase ABC digestion was determined by descending paper chromatography using C4, C6, and unsulfated oligosaccharide standards (Saito et al., 1968). The absence of iduronic acid residues within these chains was confirmed by equivalent sensitivity of chondroitin/dermatan sulfate to chondroitinase ABC (Sigma Chemical Co.), which digests chondroitin sulfate and dermatan sulfate and chondroitinase AC II (Sigma Chemical Co.), which digests only chondroitin sulfate. Detergent-extracted 35S-macromolecules resistant to the effects of nitrous acid and chondroitinase ABC digestion were considered as 35S-glycoproteins (Brown et al., 1981), and this population never exceeded 10% of any extract examined.
Role in Melanoma Cell Motility and Invasion

Cell Surface Chondroitin Sulfate Proteoglycans Play a Role in Melanoma Cell Motility and Invasion

Three-dimensional gels composed of type I collagen were used to monitor melanoma cell invasion. Approximately 77% of highly metastatic mouse melanoma cells applied to each invasion gel invaded these structures to a maximum level of 100 μm by the third day of incubation. Of the total cells that invaded these gels, ~30% penetrated to the first level (20 μm), while the remaining levels contained progressively decreasing numbers of melanoma cells (Fig. 1a, solid bars). To evaluate the potential role of CSPGs in mediating invasive behavior, melanoma cell invasion was observed in the presence of increasing concentrations of β-D-xyloside. Melanoma cell invasion was inhibited by β-D-xyloside in a concentration-dependent fashion (Fig. 1b), with ~80% inhibition observed at the highest concentration of β-D-xyloside tested (1.0 mM). The number of cells detected at each level of the invasion gel was drastically reduced in the presence of 1.0 mM β-D-xyloside (Fig. 1a, dotted bars). No inhibition was observed in the presence of α-D-xyloside (Fig. 1a, cross-hatched bars), an inactive analogue of β-D-xyloside that does not interfere with CSPG synthesis (Robinson et al., 1975). The inhibitory effects of β-D-xyloside were predominantly at the level of entry of these cells into the gels, since the depth of the furthest moving cells in the presence of β-D-xyloside was relatively unaltered compared to untreated cells.

To determine whether or not β-D-xyloside was cytotoxic, melanoma cells in culture were preincubated with 1.0 mM β-D-xyloside for 48 h, released and seeded onto the gels in the absence of additional β-D-xyloside. The number of cells invading per cm² was not significantly different for cells pretreated with β-D-xyloside (3,431 ± 138) as for cells that had not been exposed to this drug (4,052 ± 500). Furthermore, regardless of β-D-xyloside treatment, cells were determined to be 95% viable by trypan blue exclusion and protein synthesis was not affected (data not shown). Collectively, these results indicate that β-D-xyloside-mediated inhibition of melanoma cell invasion is reversible and not due to a cytotoxic effect of the drug.

To further evaluate the role of cell surface CSPG in melanoma cell invasion, the effects of β-D-xyloside on melanoma cell motility were examined in Boyden chamber type migration assays. Melanoma cell motility in response to type I collagen or fibronectin was inhibited by 50% in the presence of 1.0 mM β-D-xyloside (Fig. 2a) and not affected by the inactive analogue, α-D-xyloside (data not shown). In contrast, β-D-xyloside did not affect melanoma cell adhesion to type I collagen- or fibronectin-coated substrata (Fig. 2b). Also, by directly measuring the cytoplasmic area of adherent cells, we...
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Figure 2. β-D-Xyloside inhibits melanoma cell migration, but not adhesion or spreading on adhesion promoting proteins of the extracellular matrix. Mouse melanoma cells were pretreated for 48 h without (solid bars) or with 1 mM β-D-xyloside (cross hatched bars). (A) Cells were examined for the ability to migrate in the Boyden Chamber Assay in response to 1 µg/ml of type I collagen or fibronectin. (B) Cells radiolabeled with 3H-thymidine for the final 18 h before the assay were released from the flask with EDTA, and examined for the ability to adhere on substrata coated with 10 ng/ml of type I collagen or fibronectin. (C) Cell spreading on 10 µg/ml type I collagen or fibronectin was quantitated by directly measuring the cytoplasmic area of adherent cells. Data represent the mean percentage of triplicate determinations plus or minus the standard errors of the means.

Figure 3. Removal of cell surface CSPG by chondroitinase ABC inhibits melanoma cell motility, but not adhesion on type I collagen or fibronectin. Mouse melanoma cells were pretreated for 15 min with 0 (solid bars) or 0.1 U/ml chondroitinase ABC (cross hatched bars). (A) Cells were examined for the ability to migrate in a Boyden chamber assay in response to 10 µg/ml type I collagen or fibronectin. (B) Cells radiolabeled with 3H-thymidine, were examined for the ability to adhere to substrata coated with 10 µg/ml type I collagen or fibronectin.

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The inhibition of cell motility observed in the presence of chondroitinase ABC (0.1 U/ml) was inhibited by 90% on type I collagen and by 30% on fibronectin, suggesting an important role for CSPG in mediating motile behavior on ECM components. As was observed for β-D-xyloside pretreatment, melanoma cell adhesion to type I collagen and fibronectin was not inhibited in the presence of chondroitinase ABC (Fig. 3 b). The inhibition of motility observed in the presence of chondroitinase ABC was attributed to the action of the chondroitinase ABC enzyme, since proteases were not detectable in the enzyme mixture (data not shown, see Materials and Methods). Thus, both β-D-xyloside and chondroitinase ABC treatments inhibited melanoma cell motility but not cell adhesion or spreading on type I collagen, suggesting that CSPG may play a role in melanoma cell motility and invasion.
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**Figure 4.** Purification of detergent-extracted 35S-proteoglycans by HPLC-DEAE chromatography. (A) 35S-proteoglycans were extracted with 1% Triton X-100 and applied to an HPLC-DEAE column in DEAE buffer (50 mM Tris, pH 7.0, 6 M urea, 0.2% CHAPS, 0.1 M NaCl) and eluted with a NaCl gradient generated by HPLC. Radioactivity was monitored for each 1-ml fraction (open squares) and the salt gradient was monitored by conductivity measurements (solid diamonds). (B) The fractions eluting at 0.38 M NaCl (Fig. 4 A) were pooled (as indicated by the bar) and rechromatographed on the same column to insure maximum purity prior to further characterization.

at the level of cellular translocation, rather than as a primary cell adhesion receptor.

**β-D-Xyloside Alters CSPG Production by Highly Metastatic Melanoma Cells**

Considering our results indicating a role for cell surface CSPG in mediating melanoma cell motility and invasion, PGs synthesized by mouse melanoma cells were isolated and partially characterized. PGs may be associated with an adherent cell as integral or peripheral membrane components, or they may be deposited into the ECM (Hook et al., 1984; Yanagashita and Hascall, 1984). We followed an extraction protocol to preferentially isolate cell surface integral membrane components (Yanagashita et al., 1987). Approximately 20% of the 35S-PG could be extracted by treatment of the cell cultures with low levels of heparin (50 μg/ml), indicating that these molecules were noncovalently associated with the plasma membrane or the ECM (Hook et al., 1984). The majority (65%) of the 35S-PGs were extracted with nonionic detergent, comprising those macromolecules intrinsically associated with the cell surface or with intracellular compartments. The remaining 15% of 35S-PGs were detergent insoluble and required guanidine-HCl for solubilization, suggesting that they were associated with the detergent-resistant cytoskeleton or ECM.

To further characterize melanoma cell surface CSPG, detergent extracts of 35SO4-labeled melanoma cell cultures were purified twice by HPLC-DEAE column chromatography. The initial chromatograph of this extract contained three partially resolved peaks as shown in Fig. 4 A. These peaks were pooled and rechromatographed on HPLC-DEAE, as shown for the material eluting at 0.38 M NaCl (Fig. 4 b). 35S-CSPG eluted exclusively at 0.38 M and consisted of 85-90% chondroitin sulfate, as determined by sensitivity to chondroitinase ABC and resistance to nitrous acid. The remaining two peaks contained 35S-glycoproteins (0.21 M) or 35S-HSPGs (0.32 M) and were not further characterized in these studies.

HPLC-DEAE-purified CSPG accounted for 48% of the total detergent-extracted PG and contained exclusively chondroitin-4-sulfate, determined by descending paper chromatography, with no iduronate modifications, as indicated by >95% sensitivity of nitrous acid–resistant molecules to either chondroitinase ABC or AC. 35S-CSPG eluted from a Sepharose CL-6B column between K_σ of 0.2 (Fig. 5). 35S-Chondroitin sulfate was released from the PG by alkaline borohydride treatment. This treatment shifted the elution volume from a Sepharose CL-6B column to a K_σ of 0.55 (Fig. 5), confirming that the 35S-radiolabel was incorporated into O-linked carbohydrates.

The proportion of 35S-PG in detergent extracts of cells treated with β-D-xyloside was similar (i.e., 65%) to that obtained from untreated cell cultures, as determined by the
Figure 6. β-D-xyloside alters size distribution of detergent-extracted 35S-labeled macromolecules. Detergent-extracted 35S-proteoglycans were suspended in CL-6B buffer (0.5 M sodium acetate containing 0.2% CHAPS, pH 7.0) and applied to a 0.9 x 110 cm Sepharose CL-6B column. The CL-6B elution profiles are shown of 35S-labeled macromolecules from detergent-extracts of melanoma cells cultured in the absence (A) or presence (B) of 1.0 mM β-D-xyloside. The eluting peaks were pooled and analyzed for GAG type by nitrous acid or chondroitinase ABC digestions as described in Materials and Methods.

amount of radioactivity recovered in detergent extracts on a per cell basis (data not shown). However, the hydrodynamic properties of DEAE-purified PG from detergent extracts of melanoma cells treated with or without 1 mM β-D-xyloside were markedly different. Detergent-extracted 35S-PG from untreated or treated cell cultures were batch eluted on DEAE, pooled, and compared by Sepharose CL-6B chromatography. 35S-PG from untreated cells eluted from a Sepharose CL-6B column as two partially overlapping peaks at Kav 0 and 0.2, containing a mixture of CSPG and HSPG (Fig. 6 a). In contrast, for PG isolated from β-D-xyloside-treated cell cultures, the 35S-macromolecules eluting at Kav 0–0.2 contained HSPG, determined by 95% nitrous acid sensitivity, whereas chondroitin sulfate eluted at Kav 0.6 (Fig. 6 b). Alkaline borohydride treatment of β-D-xyloside-initiated chondroitin sulfate did not alter the Kav of the eluting material on a Sepharose CL-6B column (not shown), confirming that the chondroitin sulfate synthesis in the β-D-xyloside–treated cultures was uncoupled from core protein synthesis. Furthermore, the amount of nitrous acid–sensitive 35S-proteoglycans recovered from unfraccionated cell extracts of either culture condition differed by <10% (data not shown), suggesting that HSPG synthesis was not altered in the presence of β-D-xyloside. These results demonstrate that 1.0 mM β-D-xyloside effectively uncoupled chondroitin sulfate synthesis from the core protein, while the attachment of heparan sulfate to the core protein was essentially uninterrupted.

Detergent-extracted CSPG Produced by Melanoma Cells Binds to Type I Collagen and Has Properties Consistent with Plasma Membrane Intercalation

The above results strongly support a role for melanoma cell
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Figure 8. Detergent-extracted murine melanoma CSPG binds Octyl Sepharose. (A) HPLC-DEAE-purified $^{35}$S-CSPG was resuspended in Octyl Sepharose buffer (4.0 M Guanidine HCl, 20 mM Tris, pH 6.8) and applied to a 5-ml Octyl Sepharose column at a flow rate of 0.5 ml/min. Hydrophobic CSPG were then eluted with a linear gradient of Triton X-100 from 0 to 0.5% in Octyl Sepharose buffer. Fractions of 2 ml each were analyzed for $^{35}$S radioactivity (open squares) by liquid scintillation and percent Triton X-100 by absorbance at 280 nm (solid diamonds). (B) HPLC-DEAE-purified $^{35}$S-CSPG was briefly digested with trypsin before Octyl Sepharose chromatography.

surface CSPG in type I collagen-mediated melanoma cell motility and invasion. By utilizing a solid phase binding assay, containing type I collagen coated onto microtiter wells, we determined that DEAE-HPLC-purified CSPG bound to type I collagen in a concentration dependent and saturable fashion (data not shown). HPLC-DEAE-purified CSPG also bound to a type I collagen affinity column in the presence of 0.5% CHAPS and was eluted from the column by 0.4 M NaCl (Fig. 7 a). The binding of $^{35}$S CSPG was specific to type I collagen since it failed to bind to an affinity column prepared without type I collagen. To further evaluate the mechanism by which CSPG bound to type I collagen, chondroitin sulfate released from the protein core by alkaline $\beta$-elimination and treated with nitrous acid to remove any contaminating heparan sulfate, was applied to the type I collagen affinity column. The nitrous acid-resistant chondroitin sulfate bound to the type I collagen affinity column and eluted as a broad peak with 0.1-0.4 M NaCl (Fig. 7 b), which was much more heterogenous that that observed for intact CSPG. These studies demonstrate that mouse melanoma cell surface CSPG binds directly to type I collagen and that this interaction is mediated, at least in part, by chondroitin sulfate.

Hydrophobic chromatography has been used by other investigators to identify potential intercalated cell surface proteoglycans (Yanagashita et al., 1987). Approximately 80% of detergent-extracted HPLC-DEAE-purified $^{35}$S-CSPG bound to an Octyl Sepharose column and was eluted with concentrations of Triton-X-100 that were close to the critical micellar concentration of this detergent (Fig. 8 a). In contrast, detergent-extracted CSPG that had been treated briefly with low levels of trypsin failed to bind this column (Fig. 8 b). These results are consistent with the hypothesis that a hydrophobic domain within the detergent-extracted CSPG protein core mediates binding to the hydrophobic column. Importantly, $^{35}$S-chondroitin sulfate macromolecules isolated from $\beta$-d-xylosyl cultures also failed to bind this column (not shown), further demonstrating that $\beta$-d-xylosyl treatment prevents the expression of intact cell surface CSPG in mouse melanoma cells.

To determine the apparent molecular weight of the mouse melanoma CSPG core protein, detergent-extracted, HPLC-purified $^{125}$I-labeled CSPG was digested with chondroitinase ABC and analyzed by 6-15% SDS gel electrophoresis under nonreducing conditions (Fig. 9, lane A). A single CSPG core protein was observed at $\sim$110 kD by autoradiography, with a slight but noticeable increase in molecular mass observed upon reduction of this protein (not shown). No radioactivity was observed in lanes that contained heparatinase-digested $^{125}$I-CSPG (Fig. 9, lane B), undigested $^{125}$I-CSPG (Fig. 9, lane C), or chondroitinase ABC (Fig. 9, lane D). To confirm that the 110-kD band was a core protein of CSPG, chondroitinase digests of melanoma CSPG from similar gels were electroblotted onto nitrocellulose and probed with a polyclonal antibody that recognizes the unsaturated bonds of chondroitin sulfate-associated uronic acid residues that remain after chondroitinase ABC digestion (Couchman et al., 1985). Such blots indicate a single band at $\sim$110 kD specifically detected by the antibody (Fig. 10 A, lane 2). The specificity of the reaction is indicated by the failure of this antibody to recognize undigested CSPG (Fig. 10 A, lane 1) or chondroitinase ABC (Fig. 10 A, lane 3).

The relationship of mouse melanoma CSPG to other previously described cell surface PGs was evaluated by probing duplicate Western blots with antibodies against the human melanoma proteoglycan that has a 250-kD core protein (Bumol et al., 1984) and CD44, an 80-95-kD glycoprotein that may contain chondroitin sulfate modifications (Jalkanen et al., 1988; Brown et al., 1991). IM7, an anti-CD44 mAb (Picker et al., 1989) recognizes a single band at $\sim$110 kD in the lane containing chondroitinase-digested CSPG (Fig. 10 B, lane 4), suggesting that murine melanoma CSPG is immunologically related to CD44. No immunological reactivity was observed in lanes containing undigested CSPG (Fig. 10 B, lane 5) or chondroitinase ABC (Fig. 10 B, lane 6). Similar results were obtained with four additional mAbs, KM81, KM114, KM201, and KM703, that detect different epitopes on CD44.
Chondroitinase-digested CSPG; (3 and 6) chondroitinase ABC. (Picker et al., 1989). (Lanes 1 and 5) undigested CSPG; (2 and 4) digested CSPG; (B) IM7, a rat mAb against CD44

that recognizes the unsaturated bonds of chondroitin sulfate-associated uronic acid residues that remain after chondroitinase ABC digestion (Couchman et al., 1985) or (B) IM7, a rat mAb against CD44 (Picker et al., 1989). (Lanes 1 and 5) undigested CSPG; (2 and 4) chondroitinase-digested CSPG; (3 and 6) chondroitinase ABC.

Discussion

Our studies demonstrate an important role for a cell surface CD44-related CSPG in mouse melanoma cell motility and invasion into type I collagen matrices. By pharmacologically blocking mouse melanoma cell production of CSPG with β-D-xyloside, we observed a corresponding decrease in melanoma cell migration and invasive behavior on type I collagen.

Several lines of evidence from other investigators support the hypothesis that cell surface CSPG contributes to the motile behavior of normal and transformed cells. Wounded endothelial cell cultures have been observed to rapidly change proteoglycan production from heparan sulfate to chondroitin sulfate coincident with the onset of motility (Kinsella and Wight, 1986). CSPGs have also been implicated in regulating the migration of cardiac mesenchymal cells (Funderburg and Markwald, 1986) and neural crest cells (Perris and Johnson, 1987).

Figure 9. Identification of 110-kD core protein of mouse melanoma CSPG by autoradiography. [125I]-labeled DEAE-HPLC purified CSPG was digested with chondroitinase ABC and analyzed by 6-15% SDS-PAGE under nonreducing conditions. (Lane A) chondroitinase ABC digested CSPG; (B) heparatinase digested CSPG; (C) undigested CSPG; (D) chondroitinase ABC.

Our studies demonstrate an important role for a cell surface CD44-related CSPG in mouse melanoma cell motility and invasion into type I collagen matrices. By pharmacologically blocking mouse melanoma cell production of CSPG with β-D-xyloside, we observed a corresponding decrease in melanoma cell migration and invasive behavior on type I collagen.

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Cell surface CSPG does not appear to be a primary melanoma cell adhesion receptor for type I collagen, since cell adhesion and spreading on type I collagen were unaffected by β-D-xyloside or chondroitinase ABC treatments. Other cell surface receptors that may mediate the adhesion of moving cells include integrins, the major family of receptors known to mediate cell adhesion (Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990; Humphries, 1990).

Cell surface HSPGs have also been shown to mediate cell attachment to ECM proteins (Lark et al., 1985; LeBaron et al., 1988; McCarthy et al., 1986; Woods et al., 1986; Rogers et al., 1987; Saunders and Bernfield, 1988). HSPGs have been associated with newly formed adhesions of adherent cells (Lark et al., 1985), while older focal adhesions, found at the trailing edge of a moving cell, are primarily composed of CSPG (Culp et al., 1978). While certain cell surface HSPGs and integrins may be involved in cell motility by promoting cell adhesion and spreading at the leading edge of a moving cell, our data is consistent with the hypothesis that cell surface CSPG may disrupt these adhesive contacts and promote the release of the trailing edge of a moving cell, as has been previously proposed by Culp et al. (1978).

The molecular mechanism by which mouse melanoma CSPG modulates cell adhesion during cell motility has not been clearly defined. Several studies have indicated that CSPG, isolated from a wide variety of sources, exerts antagonistic effects on cell adhesion to ECM proteins such as fibronectin, collagen, and laminin (Knox and Wells, 1979;...
Rich et al., 1981; Brennan et al., 1983; Rosenberg et al., 1985; Yamagata et al., 1989). CSGP may interfere with cell attachment either by competing with other cell surface receptors for binding to ECM molecules at the GAG binding sites and/or by masking the integrin binding sites (Ruoslahti, 1988). By weakening cell adhesion to other ECM components, CSGP may participate in cell motility by facilitating cell detachment (Culp et al., 1986; Ruoslahti, 1988). In this regard, the ability of CS on the surface of melanoma cells to bind type I collagen may be important for bringing cell surface CSGP into a close association with other adhesion receptors such as integrins. The close proximity of cell surface CSGP to other cell adhesion receptors may serve to disrupt the association of receptors at that site of the cell membrane and subsequently alter the adhesive contacts. Alternatively, mouse melanoma CSGP could serve to transmit additional signals to the interior of the cell, thereby indirectly modulating cell adhesion and migration (Yamagata et al., 1989). Further study of the molecular and cellular basis of cell surface CSGP interactions with ECM components will be required to understand the role of cell surface CSGP in melanoma cell motility and invasion.

In addition to demonstrating an immunological relationship, the structural and functional properties of mouse melanoma CSGP are similar to CD44, a cell surface molecule that has been implicated in mediating cell-cell and cell-ECM interactions (Gallatin et al., 1989; Haynes et al., 1989; Jalkanen et al., 1988; Miyake et al., 1990). Although the 110-kD core protein of mouse melanoma CSGP is slightly larger than the well-characterized 80–95-kD CD44 glycoprotein, larger forms of CD44 have recently been identified that have various carbohydrate modifications, such as CS addition (Jalkanen et al., 1988; Brown et al., 1991), or result from alternative splicing of mRNA coding for CD44 (Brown et al., 1991; Günthert et al., 1991). The class III ECM receptor (ECMRIII [Wayner and Carter, 1987]), recently identified as CD44 (Gallatin et al., 1989), has been shown to bind to collagen, although it does not directly mediate cell adhesion (Wayner, 1987), similar to the functional properties currently identified for mouse melanoma CSGP. Furthermore, CD44 has been shown to be associated with the cytoskeleton (Jacobson et al., 1984; Kalomiris and Bourguignon, 1988) and to play a role in cell movement (Jacobson et al., 1984). Importantly, a variant form of CD44, containing an additional extracellular domain, has recently been implicated in mediating the metastatic behavior of certain rat carcinoma cell lines (Günthert et al., 1991). Additional studies that further define the structural similarities of the mouse melanoma CSGP core protein and CD44, as well as studies of this particular CSGP in low metastatic counterparts of the K1735 melanoma and normal melanocytes, will help to elucidate the mechanism by which mouse melanoma CSGP mediates tumor cell motility and invasion.

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