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# Cell surface CD44-related chondroitin sulfate proteoglycan is required for transforming growth factor-β-stimulated mouse melanoma cell motility and invasive behavior on type I collagen

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## SUMMARY

**Tumor cell metastasis involves a complex series of events, including the adhesion, migration and invasive behavior of tumor cells on components of the extracellular matrix. Multiple cell surface receptors mediate interactions with the surrounding extracellular matrix and thereby influence cell adhesion, motility and invasion. We have previously described a cell surface CD44-related chondroitin sulfate proteoglycan on highly metastatic melanoma cells. CD44-chondroitin sulfate proteoglycan was shown to be important in melanoma cell motility and invasive behavior on type I collagen matrices. In our current studies, the role of cell surface CD44-chondroitin sulfate proteoglycan in collagen-mediated mouse melanoma cell migration and invasive behavior is further evaluated using transforming growth factor-β1. We report that transforming growth factor-β1 stimulates the migratory and invasive behavior of mouse melanoma cells on type I collagen. Transforming growth factor-β1 stimulated cell surface CD44-chondroitin sulfate proteoglycan synthesis in mouse melanoma cells, specifically through an upregulation of chondroitin sulfate production, while the expression of CD44-chondroitin sulfate proteoglycan core protein was not affected. Furthermore, transforming growth factor-β1-mediated enhancement of cell polarity, migration and invasive behavior on type I collagen gels was markedly inhibited in the presence of β-D-xyloside, an agent that blocks chondroitin sulfate addition to the core protein. Collectively, our findings indicate that mouse melanoma cell surface CD44-chondroitin sulfate proteoglycan is required for transforming growth factor-β1-enhanced cell motility and invasion, and that CD44-chondroitin sulfate proteoglycan may play a role in forming and/or maintaining a dominant leading lamella, which is required for efficient locomotion.**

Key words: cell surface proteoglycans, motility, invasion, CD44

## INTRODUCTION

Cell migration is fundamentally important to embryogenesis and plays a major role in many normal and pathological processes, such as tumor cell invasion and metastasis. Tumor cell metastasis is known to involve a complex series of events, including the adhesion and migration of tumor cells on extracellular matrix (ECM) components (Liotta et al., 1983). The ECM of tissues and basement membrane can facilitate tumor cell invasion by promoting directional cell movement by haptotactic and contact guidance mechanisms (McCarthy et al., 1985). While originally considered primarily as structural components of the ECM, several collagen types have been shown to directly mediate the adhesion and motility of many normal and malignant cell types (Liotta et al., 1983; Aumailley and Timpl, 1986; Rubin et al., 1981; Dedhar et al., 1987; Herbst et al., 1988; Chelberg et al., 1989, 1990). Understanding the mechanism of ECM-directed cell migration and the cellular macromolecules involved in this process will be extremely valuable for evaluating disease processes.

We have previously demonstrated that mouse melanoma CD44-related chondroitin sulfate proteoglycan (CSPG) plays an important role in collagen-mediated cell motility and invasion (Faassen et al., 1992). By blocking K1735-M4 mouse melanoma cell surface CSPG production with *p*-nitrophenyl β-D-xylopyranoside (β-D-xyloside; Schwartz, 1977), we observed a corresponding decrease in collagen-mediated cell motility and invasive behavior, while melanoma cell adhesion on collagen coated substrates was not affected. These studies suggested that mouse melanoma cell surface CSPG may be important in cell

migration, but not as a primary cell adhesion receptor. In support of these observations, CSPGs have been implicated in the motile behavior of various normal and transformed cell types (Kinsella and Wight, 1986; Funderburg and Markwald, 1986; Perris and Johansson, 1987), however, the precise mechanisms through which CSPG mediates cell motility have not been defined. Certain lines of evidence suggest that CSPG disrupts cell adhesion (Culp et al., 1986; Ruoslahti, 1988) and thereby facilitates the detachment of the trailing edge of a migrating cell. By contrast, recent studies have implicated melanoma cell surface CSPG in mediating early recognition events in cell adhesion (Iida et al., 1992) consistent with previous data localizing the large human melanoma CSPG to cell surface microspikes (Garrigues et al., 1986). Our current studies further evaluate the role of CSPG in cell migration on ECM components.

In a variety of cell types, CSPG synthesis has been shown to increase in response to transforming growth factor-β1 (TGF-β), a polypeptide growth factor that induces a multiplicity of biological activities (reviewed by Massague, 1990). TGF-β has been shown to elevate the expression of PG core proteins as well as the size or total mass of GAG chains attached to them (Bassols and Massague, 1988; Chen et al., 1987; Rasmussen and Rapraeger, 1988; Rapraeger, 1989; Uhlman et al., 1990). Previous work from our group has demonstrated that TGF-β enhances the invasive behavior of human pulmonary adenocarcinoma cells into type I collagen matrices (Mooradian et al., 1992), although the mechanism for this stimulation has not been elucidated. In this report, we demonstrate that TGF-β also stimulates the migratory and invasive behavior of highly metastatic K1735-M2 mouse melanoma cells on type I collagen gels. Furthermore, TGF-β upregulates the synthesis of cell surface CD44-CSPG in mouse melanoma cells, specifically through the stimulation of chondroitin sulfate (CS) production, while levels of CD44-related PG core protein expression remain unaltered. Finally, it has been observed that TGF-β-mediated stimulation of cell polarity, migration and invasive behavior is dramatically reduced in the presence of β-D-xyloside. These findings indicate that cell surface CD44-CSPG is required for TGF-β-stimulated melanoma cell motility and invasive behavior, and suggest that CD44-CSPG may be important for forming and/or maintaining dominant pseudopods in a leading lamella, which are required for efficient cell locomotion.

## MATERIALS AND METHODS

### Cell culture

A highly metastatic clone (M2) of the K1735 mouse melanoma was generously provided by Dr I. J. Fidler (M. D. Anderson Hospital Cancer Center, Houston, TX). This melanoma cell line was maintained by in vitro culture in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated calf serum (Sigma Chemical Co.). The number of in vitro passages was limited to eight in order to minimize phenotypic drift.

### Cell invasion and motility assays

Gels composed of bovine type I collagen fibrils (Collagen Corp., Palo Alto, CA) with DMEM, 10% PBS, 0.225% $NaHCO_3$ were prepared as previously described (Faassen et al., 1992) in 6-well plates (Costar, Cambridge, MA), and equilibrated for at least two times four hours with a 2 ml overlay of DMEM (pH 7.4). Cells were released with trypsin (0.25%)/EDTA (1 mM), resuspended in DMEM supplemented with 2.5% heat-inactivated calf serum, added to the top of the gels at $1\times10^5$ cells per well and incubated at 37°C in a humidified 5% $CO_2$ incubator. Cell invasion was measured after 24 and 48 hours, as described previously (Faassen et al., 1992), using an inverted phase-contrast microscope (Nikon Diaphot) at ×200 magnification. The number of invading cells was determined by counting the number of cells in five randomly selected fields at increasing depths within the gels until no additional cells were detected. Data are presented as the total number of cells penetrating the gel per $cm^2$ of surface area. The number of cells which remained adherent to the top of the collagen gel and did not penetrate the gel surface were counted and added to the number of invading cells to determine the total number of cells in the invasion assay system. Results are reported as the mean, plus or minus standard error of the mean, of triplicate gels.

Cell motility was measured in a Boyden chamber assay, as previously described (Faassen et al., 1992), by precoating the underside of 8 μm pore-size polyvinyl pyrrolidone-free polycarbonate filters (Nucleopore, Pleasanton, CA) with type I collagen (100 μg/ml). Results are reported as the mean number of migrated cells per $mm^2$, plus or minus the standard error of the mean ($n$=12).

### Proteoglycan extraction, purification and characterization

Melanoma cell surface proteoglycans (PGs) were labeled with [$^{35}$S]sulfate, extracted with detergent, and purified by anion exchange chromatography, as described previously (Drake et al., 1992; Faassen et al., 1992). Total [$^{35}$S]sulfate incorporation into detergent extracts, determined after recovery of radiolabeled material from a DEAE column, is reported as mean values of $^{35}$S d.p.m./cell, plus or minus standard error of the mean of triplicate determinations. The heparan sulfate and chondroitin sulfate content of $^{35}$S-labeled macromolecules, determined by sequential nitrous acid deaminative cleavage and chondroitinase ABC (Seikagagu America Inc., Rockville, MD) treatment, respectively, as previously described (Brown et al., 1981), is reported as the mean [$^{35}$S]sulfate d.p.m. values plus or minus the standard error of the mean of triplicate determinations for equivalent numbers of cells. [$^{35}$S]sulfate-labeled CSPG was purified by HPLC-DEAE chromatography, eluting with 0.4 M NaCl on the HPLC-DEAE column, and was routinely rechromatographed over the column a second time to ensure 85-95% purity of the CS, verified by chondroitinase ABC sensitivity and nitrous acid resistance, as described previously (Faassen et al., 1992).

To evaluate the relative amount of sulfate per CS disaccharide, melanoma cells were labeled with [$^{35}$S]sulfate, as described previously (Faassen et al., 1992), with the addition of 33 μCi/ml 6-$^3$H-labeled D-glucosamine hydrochloride (specific activity, 40 Ci/mmol; ICN Biomedicals, Irvine CA). $^{35}$S/$^3$H-labeled CS disaccharides were recovered following chondroitinase ABC digestion of HPLC-DEAE purified CSPG, and analyzed by descending paper chromatography using C4, C6, and unsulfated oligosaccharide standards (Saito et al., 1968). The absence of iduronic acid residues within CS chains was confirmed by equivalent sensitivity to chondroitinase ABC and chondroitinase ACII (Sigma Chemical Co.).

The hydrodynamic properties of HPLC-DEAE purified [$^{35}$S]sulfate-PG were evaluated by gel filtration on a 0.9 cm×100 cm Sepharose CL-4B column, equilibrated and eluted with 0.05 M sodium acetate, pH 7.0, containing 4 M guanidine, 1% Triton X-100 and 0.04% sodium azide at a flow rate of 3 ml/h (Faassen et al., 1992). The hydrodynamic properties of alkaline borohydride-

released CS chains (Faassen et al., 1992; Oegema et al., 1979) were evaluated by gel filtration on a Sepharose CL-6B (Sigma Chemical Co.) column, equilibrated and eluted with 0.5 M sodium acetate, pH 7.0, containing 0.2% CHAPS at a flow rate of 3 ml/h.

To examine the effect of TGF-β on CSPG core protein synthesis, mouse melanoma cells that had been treated (or not) for 24 hours with TGF-β were metabolically labeled overnight (12-14 hours) with $^{35}$S-labeled amino acids (L-methionine and L-cysteine, specific activity 1000 Ci/mmol, ICN Biomedicals, Irvine, CA) in RPMI 1640 containing 0.5 mM sulfate, without methionine or cysteine. To determine total $^{35}$S-labeled amino acid incorporation into cellular proteins, 5 μl samples (in triplicate) of cellular extracts were added to 100 μl 1% BSA plus 1 ml ice cold TCA (10%), and incubated on ice for 30 min. Precipitated proteins were trapped on glass fiber filters under vacuum, washed extensively with TCA (10%), and radioactivity was quantitated by liquid scintillation (Beckman LS3801). Results are reported as mean values of $^{35}$S d.p.m./cell, plus or minus standard error of the mean of triplicate determinations. In order to determine the radiolabel incorporation into CSPG, CSPG was labeled with $^{35}$S-labeled amino acid and purified by methods which have previously been shown to yield a pure preparation of CD44-CSPG that gave only a single band on SDS-PAGE after chondroitinase ABC digestions and iodination (Faassen et al., 1992). Briefly, macromolecules labeled with $^{35}$S-labeled amino acids were isolated by two sequential HPLC-DEAE runs, pooled according to a NaCl elution profile, and further purified on a Sepharose CL-4B (Sigma Chemical Co.) column, as described above, and the fractions that migrated in the same region as [$^{35}$S]sulfate-labeled CSPG were pooled and counted. Results are expressed as the total $^{35}$S d.p.m. in CD44-CSPG purified from equivalent numbers of untreated or TGF-β-treated cells.

## Immunoblots and autoradiography

To evaluate CSPG core protein expression by immunoblot, HPLC-DEAE-purified CSPG was digested with chondroitinase ABC and electrophoresed on 10% SDS-PAGE under non-reducing conditions, as described previously (Faassen et al., 1992). Following electrophoresis, proteins were transferred onto Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), which was then probed with a rabbit polyclonal antibody that recognizes the unsaturated uronic acid residues that remain attached to the PG core protein after chondroitinase ABC digestion (Couchman et al., 1985; kindly provided by Dr John Couchman), as described previously (Faassen et al., 1992). To evaluate CD44 core protein expression, duplicate blots were probed with rat anti-CD44 mAb, IM7 (Picker et al., 1989; kindly provided by Dr Eugene Butcher).

## β-D-Xyloside and TGF-β pretreatment

To evaluate cell surface PGs produced by TGF-β-treated melanoma cells, cell monolayers were first treated for 20 hours with 10 ng/ml recombinant simian TGF-β1 (a generous gift from Anthony Purchio, Bristol-Myers Squibb, Seattle, WA) in DMEM containing 2.5% heat-inactivated calf serum, then treated for 18 hours with 10 ng/ml TGF-β in the [$^{35}$S]sulfate labeling medium prior to PG extraction. For the invasion assays, cells were seeded onto the gels as described below in the presence of 10 ng/ml TGF-β and/or 1.0 mM *p*-nitrophenyl-β-D-xylopyranoside (β-D-xyloside; Sigma Chemical Co.). For the surface migration assays, cells were pretreated with 1 mM β-D-xyloside for 24 hours, as described previously (Faassen et al., 1992), then seeded onto the collagen gel in the continued presence of 1 mM β-D-xyloside. TGF-β (10 ng/ml) was added to the cells in the motility assay at the time of seeding.

## Cell migration on the surface of collagen gels

To evaluate cell migration on type I collagen gels, gels were prepared in 50 mm dishes (Beckton Dickinson, Lincoln Park, NJ), as described above. These dishes snap closed to prevent air exchange. Gels were equilibrated with DMEM (no $NaHCO_3$) supplemented with 20 mM Hepes, pH 7.2, and incubated in a 37°C, humidified incubator, without $CO_2$. Cells were seeded in DMEM and 2.5% heat-inactivated calf serum containing 20 mM Hepes, so that the cells could withstand observation for 24 hours in a $CO_2$-deficient environment. The culture dish was placed on an inverted microscope (IM 35; Carl Zeiss Inc., Thornwood, NY) and maintained at a constant 37°C with an air curtain incubator (ASI 400; Carl Zeiss Inc.). A field of approximately 25 cells was randomly selected for videotaping during the first hour after seeding, imaged with a video camera (Dage-MTI model 65 Newvicon) and a monitor (Trinitron; Sony Corp. of America, New York, NY). Phase contrast images were enhanced and analyzed using a computer controlled image processing system (Gateway 2000, North Sioux City, SD; Image 1 software, Universal Imaging Corporation, West Chester, PA.). Cells were observed between 1 and 22 hours after seeding the cells on the collagen gels, and images were recorded on an optical disc recorder (Panasonic TQ-2026F; Panasonic Company, Secaucus, NJ) at 5 minute intervals. Cell migration was quantitated with Image 1 software (Universal Imaging Corporation; West Chester, PA.) by determining the coordinates of each cell position for every sixth frame (30 min intervals). The distance between each point was measured in μm and marked to record a complete cell track.

The cell tracks were characterized quantitatively by a motility coefficient, μ, which measures the tendency of cells to disperse from their initial positions, thus reflecting the efficiency of random migration. (μ is analogous to a diffusion coefficient, which is a measure of the tendency of molecules to disperse from their initial positions, thus reflecting the efficiency of Brownian motion). For cell migration on an anisotropic substratum, μ can be obtained by measuring the mean-squared displacement, $\langle d^2 \rangle$, for a set of individual cell tracks over increasing time intervals, $t$ (Gail and Boone, 1970; Othmer et al., 1988; Dunn, 1983), and fitting this data to the equation:

$$\langle d^2(t) \rangle = 4\mu(t - P(1 - exp(-t/P))). \quad (1)$$

The index, $P$, is the directional persistence time, which reflects the average time a cell persists in the same direction. Although presently no theoretical basis exists for the validity of (1) for cell migration on an anisotropic substratum, such as aligned collagen fibrils, we use (1) as a phenomenological model with μ and $P$ representing directionally-averaged parameters. Then $\langle d^2(t) \rangle$ is the mean of the squared displacements in orthogonal directions, i.e. parallel and perpendicular to the axis of fibril alignment, and μ is a directionally-averaged motility coefficient. We justify the use of (1) for cell migration on aligned fibrils by noting the excellent fit of (1) to the experimental data in Fig. 7.

Although it is possible that differences in the degree of fibril alignment may significantly affect the degree of cell migration with respect to the axis of alignment (i.e. contact guidance; Guido and Tranquillo, 1993), we expect that any enhancement of migration in that direction would correspond to a decrease in the orgthogonal direction, as demonstrated for neutrophils on oriented non-biological surfaces by Mathes and Gruler (1988) and fibroblasts in an oriented collagen gel (R. B. Dickinson, S. Guido and R. T. Tranquillo, unpublished). Since μ is a directionally-averaged index, we expect little dependence on the degree of fibril alignment. Therefore, we do not expect the observed large differences in the measured motility coefficients to be the result of any differences in the degree of fibril alignment that occur in the migration assay on the collagen gel surface.

The individual cell tracks in each field were analyzed to obtain an estimate of $<d^2(t)>$ over time intervals, $t$, of increasing length (incremented by 30 minutes, from 30 minutes up to 600 minutes). This estimate was obtained for each field by averaging the square displacement $d^2$ over equal (overlapping) intervals of time for each cell track, and over all cell tracks in the field. Repeating this procedure for increasing time intervals (30 minute increments) provided data for $<d^2>$ versus $t$. To obtain an estimate for $\mu$, this data was fitted to equation (1) by nonlinear least squares regression (Dickinson and Tranquillo, 1993). Reported errors are the standard errors in the estimates derived from the regression analysis (Seber and Wild, 1989).

## RESULTS

### TGF-β stimulates collagen-mediated melanoma cell migration and invasive behavior

Three-dimensional type I collagen gels were used to examine the effect of TGF-β on K1735-M2 mouse melanoma cell invasive behavior. Such gels have previously been used to evaluate the invasive behavior of an independently isolated and equally metastatic clonal cell line, termed the K1735-M4 melanoma cell line (Faassen et al., 1992). A dose-dependent increase in the total number of mouse melanoma cells invading the collagen gels corresponded to increasing concentrations of TGF-β, with a maximal enhancement (56%) at 10 ng/ml TGF-β, after 48 hours (Fig. 1). To verify that TGF-β-stimulated cell invasion into the collagen gels was not simply due to an increase in melanoma cell proliferation, we determined that the total number of cells in the collagen gel invasion assay after 48 hours was not significantly different in the presence or absence of TGF-β (data not shown). Thus the enhanced invasive behavior observed for the TGF-β-treated cell population was not a result of increased cell proliferation, consistent with previous studies demonstrating that TGF-β had no effect on the growth of these cells during a 48 hour period (Mooradian et al., 1990).

One of the initial stages of tumor cell invasion involves the migration of metastatic cells on ECM components. To further evaluate the effects of TGF-β on mouse melanoma cell invasion, melanoma cell motility on type I collagen was examined in the presence of TGF-β in a Boyden chamber assay. The number of cells migrating to the underside of a filter precoated with type I collagen was significantly greater for TGF-β treated cells than untreated cells (663 ± 117 and 130 ± 66 cells/mm$^2$, respectively; $n$=12, $P$<0.001). These results suggest that TGF-β may promote the haptotactic migration of melanoma cells on a collagen-coated substratum, during the process of invasion.

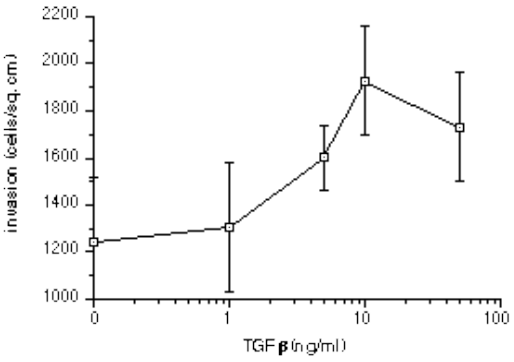


**Fig. 1.** TGF-β stimulates mouse melanoma cell invasion into type I collagen gels. The total number of cells invading the collagen gels in the absence or presence of increasing concentrations of TGF-β was determined. The data are presented as the mean number of cells invading per square cm after 48 hours (for duplicate determinations), plus or minus the standard error of the mean.

### TGF-β upregulates the synthesis of chondroitin sulfate chains on CD44-CSPG core protein

We have previously shown that cell surface CSPGs play an important role in K1735-M4 mouse melanoma cell motility and invasive behavior on type I collagen. Considering our current studies demonstrating that TGF-β stimulates collagen-mediated migratory and invasive behavior of the related K1735-M2 cells, and that TGF-β has been shown by other investigators to upregulate CSPG synthesis (Chen et al., 1987; Rasmussen and Rapraeger, 1988; Rapraeger, 1989; Uhlman et al., 1990), we wanted to evaluate the effects of TGF-β on the synthesis of mouse melanoma cell surface CSPGs. To initially determine whether TGF-β altered CSPG synthesis by these cells, [$^{35}$S]sulfate incorporation into cell surface PG was evaluated in cultures of mouse melanoma cells treated with or without TGF-β (Table 1). The ratio of heparan sulfate (HS) to chondroitin sulfate in these untreated cells was essentially identical (approximately 1:1 ratio) to that reported for the K1735-M4 cell line (Faassen et al., 1992). Total [$^{35}$S]sulfate incorporation into detergent extracts of TGF-β-treated cells was increased by 51% compared to untreated cell extracts (48.6 ± 0.9 and 32.1 ± 0.6 [$^{35}$S]sulfate d.p.m.×10$^{-3}$/cell, respectively, for triplicate determinations), as determined by the amount of radioactivity recovered in detergent extracts on a per cell basis. We then evaluated whether the increased levels of [$^{35}$S]sulfate in TGF-β-treated cell extracts was incorporated into HS- or CS-macromolecules. The amount of DEAE-purified [$^{35}$S]sulfate-labeled PG that was sensitive to nitrous acid on a d.p.m./cell basis was equivalent for cells treated with or without TGF-β, while the chondroitinase sensitive material from TGF-β-treated cells was augmented by 65% (Table 1). Thus, TGF-β treatment of melanoma cells stimulated the synthesis of [$^{35}$S]sulfate-

**Table 1. TGF-β stimulated levels of [$^{35}$S]sulfate-labeled CS-macromolecules, but did not alter levels of [$^{35}$S]sulfate-labeled HS-macromolecules**

| | −TGF-β1 | +TGF-β1 |
|---|---|---|
| CSPG (degraded by chondroitinase ABC) | 101.1±1.9* | 166.6±23† |
| HSPG (degraded by nitrous acid) | 90.5±4.9 | 92.7±9.4‡ |

*d.p.m.×10$^{-3}$ ± standard error of the mean of triplicate determinations for equivalent numbers of cells.

†d.p.m. of [$^{35}$S]sulfate-labeled CS from TGF-β-treated cells is significantly higher than untreated ($P$<0.01).

‡d.p.m. of [$^{35}$S]sulfate-labeled HS was not significantly different for the two treatments.

labeled CS-macromolecules, but did not alter the levels of [$^{35}$S]sulfate-labeled HS-macromolecules.

To compare cell surface CSPGs expressed by mouse melanoma cells cultured in the presence or absence of TGF-β, [$^{35}$S]sulfate-labeled CSPG was isolated from detergent extracts of mouse melanoma cells and purified by anion exchange chromatography, as described previously (Faassen et al., 1992). The HPLC-DEAE profile illustrates the increased synthesis of [$^{35}$S]sulfate-labeled CSPG molecules for cells cultured in the presence of TGF-β (Fig. 2). To determine if the increase in TGF-β-produced [$^{35}$S]sulfate-labeled CSPG was due to more GAG per PG core protein, the hydrodynamic size of HPLC-DEAE-purified CSPG was compared for cells pretreated (or not) with TGF-β. Cell surface CSPG from TGF-β-treated cells eluted at $K_{av}$ 0.32 on a Sepharose CL-4B under dissociative conditions, compared to $K_{av}$ 0.51 for CSPG from untreated melanoma cells (Fig. 3a). Thus, TGF-β-treated cells produced cell surface CSPG with a larger hydrodynamic size than untreated cells, possibly as a result of more CS chains per core and/or longer CS chains. To examine the latter possiblity, the hydrodynamic properties of alkaline borohydride-released CS chains were examined on a Sepharose CL-6B column (Fig. 3b). In this experiment, TGF-β-produced CS eluted at $K_{av}$ 0.48, compared to $K_{av}$ 0.57 for CS from untreated cells, corresponding to 23.3 kDa and 14.6 kDa molecular masses, respectively, based on calculations by Wasteson (1971). Thus CS chains from TGF-β-treated cells were 60% larger by molecular mass, based on hydrodynamic size, than that of CS produced by untreated cells. This increase in hydrodynamic size correlates with a 65% increase in levels of CS macromolecules in TGF-β-treated cell extracts, as described above (Table 1). Although it is possible that more CS chains may be attached per CSPG core protein, the observed increase in GAG chain length alone is sufficient to account for the increase in [$^{35}$S]sulfate-labeled CSPG production that was observed for TGF-β-treated melanoma cells. Furthermore, we determined that TGF-β did not alter sulfate incorporation per CS disaccharide (data not shown) and did not induce iduronate modifications.

To evaluate the molecular mass(es) of CSPG core protein(s) produced by TGF-β-treated melanoma cells, HPLC-DEAE-purified CSPG was digested with chondroitinase ABC, electrophoresed on a 10% SDS-PAGE gel and transfered to a nylon membrane. This western blot was probed with a polyclonal antibody that recognizes the unsaturated bonds of CS-associated uronic acid residues that remain after chondroitinase ABC digestion (Couchman et al., 1985). A single diffuse band was observed at approx. 110 kDa for CSPG from TGF-β-treated cell extracts. This band was identical to that from untreated cells (Fig. 4a) and indicates that mouse melanoma cells produce a 110 kDa CSPG core protein, in the presence or absence of TGF-β, and that TGF-β does not induce the production of other CSPG core

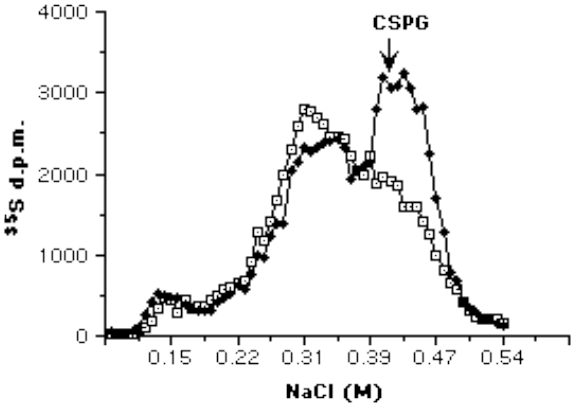


**Fig. 2.** TGF-β stimulates the synthesis of [$^{35}$S]sulfate-labeled CSPG. [$^{35}$S]sulfate-labeled PGs were extracted from cells, cultured with (solid diamonds) or without (open squares) 10 ng/ml TGF-β and applied to an HPLC-DEAE column in 50 mM Tris, pH 7.0, containing 6 M urea, 0.2% CHAPS, 0.1 M NaCl, and eluted with a NaCl gradient generated by HPLC. Radioactivity was monitored for each 1 ml fraction and the salt gradient was measured by conductivity.

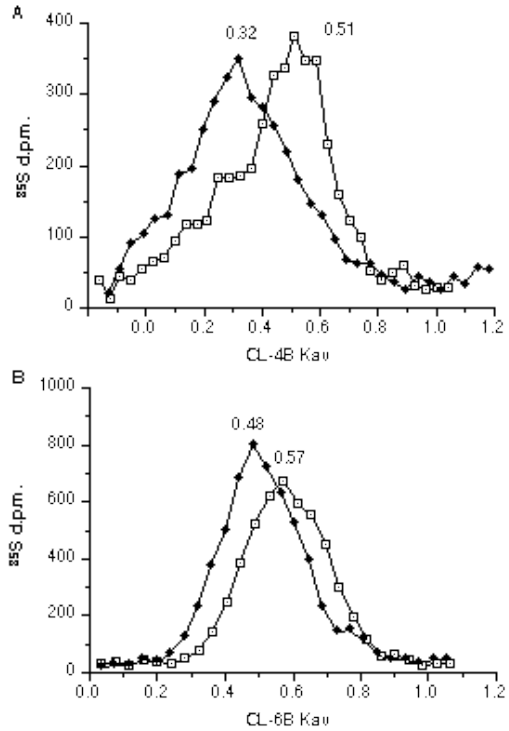


**Fig. 3.** TGF-β increases the hydrodynamic size of mouse melanoma CSPG and alkaline borohydride-released CS. Equal numbers of $^{35}$S d.p.m. of (A) HPLC-DEAE-purified CSPG or (B) alkaline borohydride released CS were loaded onto a Sepharose CL-4B column, in the presence of 4 M guanidine, 1% Triton X-100, or a Sepharose CL-6B column in the presence of 0.2% CHAPS, respectively. Elution profiles are shown of [$^{35}$S]sulfate-labeled CS-macromolecules from detergent extracts of melanoma cells cultured in the presence (solid diamonds) or absence (open squares) of 10 ng/ml TGF-β. $K_{av}$ values, indicated for the peak sample fraction, were determined by elution relative to rat chondrosarcoma chondroitin sulfate proteoglycan and glucuronolactone used to mark the column void and total volumes, respectively.

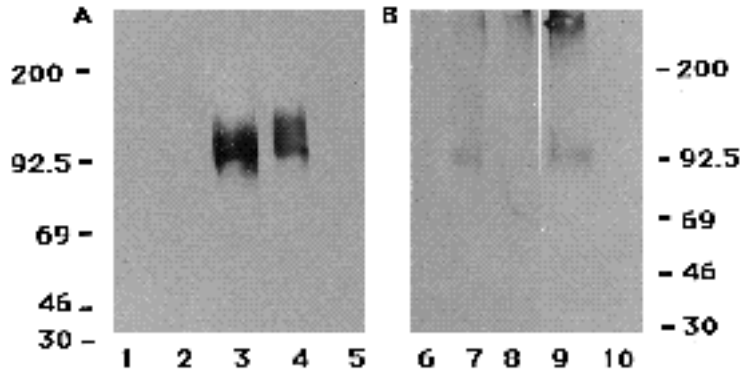


**Fig. 4.** TGF-β does not alter the synthesis of the 110 kDa core protein of mouse melanoma cell surface CD44-CSPG. DEAE-HPLC-purified CSPG was digested with chondroitinase ABC and electrophoresed under nonreducing conditions on 10% SDS-PAGE. Proteins on these gels were transferred to nylon membrane and probed with (A) 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) or (B) IM7, a rat mAb against CD44 (Picker et al., 1989). (Lanes 1 and 6) chondroitinase ABC; (lanes 2 and 8) undigested CSPG from untreated cells; (lanes 3 and 7) chondroitinase-digested CSPG from untreated cells; (lanes 4 and 9) chondroitinase-digested CSPG from TGF-β-treated cells; (lanes 5 and 10) undigested CSPG from TGF-β-treated cells. $M_r$ markers are shown at the right and left ($\times 10^{-3}$).

proteins. In addition, IM7, an anti-CD44 monoclonal antibody (Picker et al., 1989), recognized this 110 kDa CSPG core protein from both untreated and TGF-β-treated cells (Fig. 4b). Thus, exposure to TGF-β does not alter mouse melanoma cell expression of the 110 kDa CD44-related CSPG core protein.

To compare levels of CD44-CSPG core protein expression, mouse melanoma cells treated with or without TGF-β were metabolically labeled with [$^{35}$S]methionine. Incorporation of radiolabeled amino acids (cysteine and methionine) into TCA-precipitable protein was not significantly different on a per cell basis for TGF-β-treated and untreated cells (0.712 ± 0.04 and 0.765 ± 0.05 $^{35}$S-labeled amino acid d.p.m.$\times 10^{-3}$/cell, respectively, for triplicate determinations), indicating that TGF-β did not alter levels of total protein synthesis. To compare levels of CD44-CSPG core protein synthesis, metabolically labeled CSPG was purified by methods which have previously been shown to yield a pure preparation of cell surface CSPG, as indicated by a single band on SDS-PAGE after iodination (Faassen et al., 1992). Mouse melanoma cell surface CSPG labeled with $^{35}$S-labeled amino acids was isolated by HPLC-DEAE chromatography and further purified by a Sepharose CL-4B sizing column, under dissociative conditions. The levels of radiolabeled amino acids in purified CD44-CSPG from equivalent numbers of TGF-β-treated or untreated cells differed by <10% (data not shown), indicating that TGF-β did not alter levels of mouse melanoma CD44-CSPG core protein expression.

### TGF-β-stimulated melanoma cell migratory and invasive behavior on collagen gels requires cell surface CSPG

Considering that TGF-β upregulates CS synthesis on CD44-CSPG core protein, we wanted to determine whether TGF-β-stimulation of mouse melanoma cell invasion into a collagen gel (Fig. 1) is dependent upon cell surface CSPG. Melanoma cell invasion of three dimensional collagen gels was measured after 24 and 48 hours in the absence or presence of TGF-β and/or β-D-xyloside. As we had previously observed with K1735-M4 cells, the invasion of the M2 cell line was similarly sensitive to the addition of 1 mm β-D-xyloside, a concentration of the drug which completely uncouples CS from core protein synthesis (data not shown and Faassen et al., 1992). No inhibition was observed in the presence of α-D-xyloside (data not shown), an inactive analog of β-D-xyloside that does not interfere with CSPG synthesis (Robinson et al., 1975). β-D-xyloside also inhibited TGF-β-stimulated melanoma cell invasion to levels approximately 40% below the untreated cell population (Fig. 5a), similar to the level of invasion obtained with β-

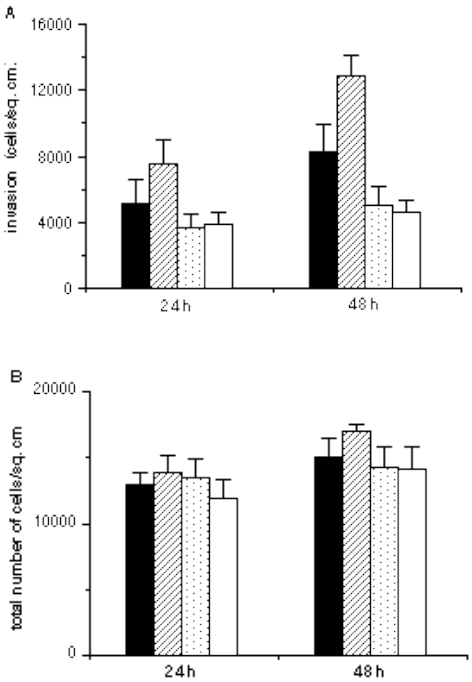


**Fig. 5.** β-D-xyloside inhibits TGF-β-stimulated mouse melanoma cell invasion of type I collagen gels. (A) The number of cells invading the gels and (B) the total number of cells in the assay in the absence (solid bars) or presence (cross hatched bars) of 10 ng/ml TGF-β, 1 mM β-D-xyloside (dotted bars) or TGF-β and β-D-xyloside (open bars) was determined. The data are presented as the mean number of total cells invading per square cm after 24 and 48 h (performed in triplicate gels), plus or minus the standard error of the mean.

D-xyloside treatment alone. These data suggest that TGF-β may be stimulating melanoma cell invasive behavior through a CSPG-dependent mechanism. The total number of cells in the collagen gel invasion assay in all treatment conditions was not significantly different (Fig. 5b), indicating that β-D-xyloside inhibited melanoma cell invasion,

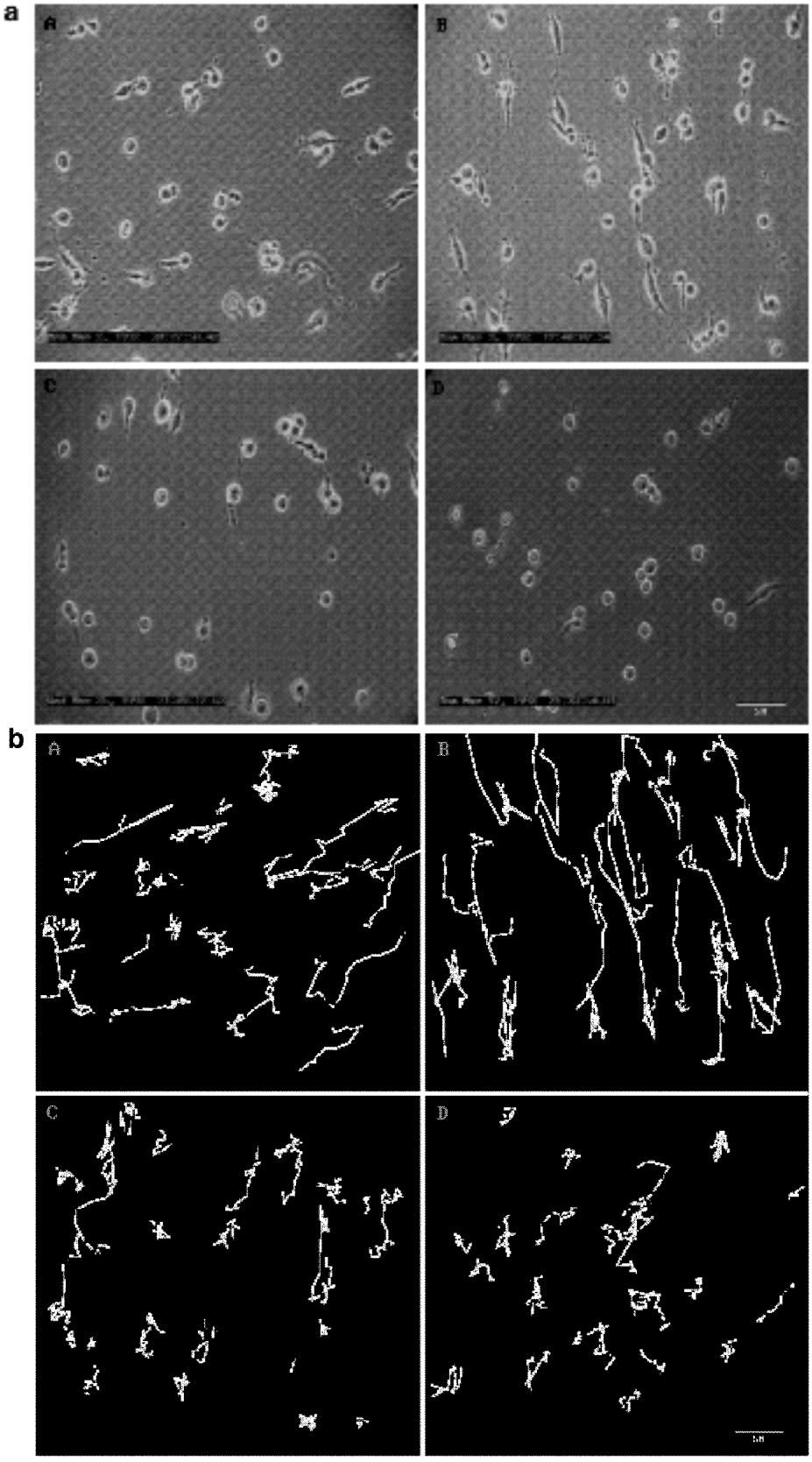


**Fig. 6.** β-D-xyloside inhibits TGF-β-stimulated melanoma cell migration on type I collagen gels. The migration of melanoma cells on type I collagen gels was observed by videomicroscopy between 1 and 22 h after seeding. The cell tracks were determined for all cells in the field using an Image 1 software analysis system (Universal Imaging Corp.) by recording the position of each cell every 30 minutes. Shown are (a) the cells on the collagen gel photographed approximately 10 hours after seeding, and (b) the cell tracks in the absence (A) or presence (B) of 10 ng/ml TGF-β, 1 mM β-D-xyloside (C) or TGF-β and β-D-xyloside (D). Bar (D), 50 μm.

but did not affect cell growth, consistent with our previous results using K1735-M4 cells (Faassen et al., 1992).

To further evaluate the mechanisms by which cell surface CSPG may be mediating melanoma cell invasion, melanoma cell migration on the surface of type I collagen gels was examined in the presence of TGF-β and/or β-D-xyloside. Although we have previously used Boyden chamber techniques to study cell migration, the current work utilizes time-lapse video microscopy to allow direct observations of melanoma cell migration on the collagen gel, and is accompanied by mathematical analysis in order to quantitatively describe the migratory behavior of a cell population.

The migration of melanoma cells on the collagen gel surface was monitored by time-lapse video microscopy beginning one hour after seeding the cells on the collagen gel, and continuing for a period of up to 22 hours. Cell paths were traced for all in focus cells in the field (Fig. 6a, approximately 25), by recording the position of each cell every 30 minutes, and determining the coordinates for each cell position using a computerized image analysis system (see Materials and Methods). Each point was connected by a line to create a discrete approximation of the cell path, or track. As observed from the cell pathway tracings, the majority of cell tracks for cells treated with TGF-β are longer than the cell tracks for the untreated cell population (Fig. 6b, A and B), consistent with the elevated migration of TGF-β-treated cells in the Boyden chamber. To evaluate the role of cell surface CSPG in TGF-β-stimulated cell motility, melanoma cells, pretreated for 24 hours with β-D-xyloside, were seeded on the collagen gels in the presence or absence of TGF-β, with the continued presence of β-D-xyloside. Qualitatively, β-D-xyloside treated cells traveled much shorter distances on the collagen gel compared to untreated cell populations, regardless of the presence of TGF-β, as observed by their truncated migration paths (Fig. 6b, C and D). The TGF-β treated cells appeared to migrate with greater directional persistence on the collagen, as illustrated by the long straight cell tracks, while the β-D-xyloside treated cells moved back and forth within a limited area.

In all culture conditions, the cells moved bidirectionally, presumably along the axis of aligned collagen fibrils, a phenomenon termed contact guidance, which has previously been observed in other collagen gel assay systems (Elsdale and Bard, 1972; Kono et al., 1990; Dunn and Ebendal, 1978). The alignment of the collagen fibrils was observed in repeated experiments under each culture condition, as well as in the absence of cells. Although this alignment occurred in an uncontrolled fashion, the index used to quantify cell migration, the direction-averaged motility coefficient, μ, derived from the cell tracks should not be strongly dependent on variations in the extent of alignment (see Materials and Methods). This index allows an objective, quantitative comparison between the migratory behavior of cells under different conditions, and measures the tendency of cells to disperse from their initial positions, thus reflecting the efficiency of cell migration.

To determine μ, the mean squared displacement $\langle d^2 \rangle$ of individual cells was plotted versus the time interval, *t*, over which the displacements occur (Fig. 7). The nonlinear regression fit of equation (1) to the experimental data is excellent for all culture conditions, and μ is estimated as one fourth the asymptotic slope of the line. As shown in Fig. 8, μ is markedly increased following treatment with TGF-β when compared to untreated cells, indicating that

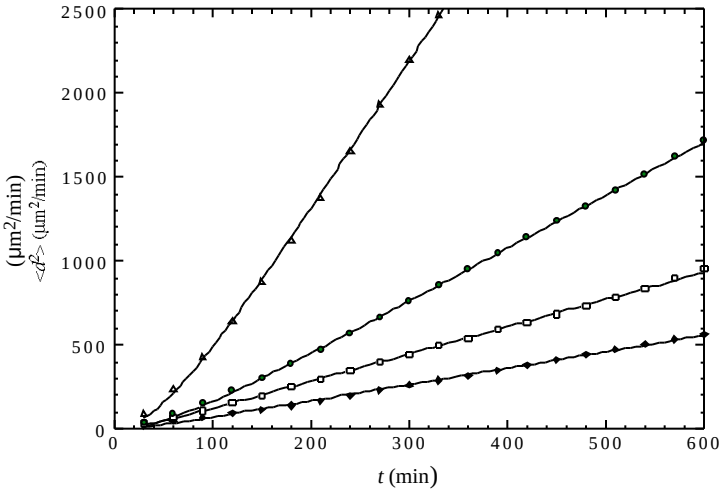


**Fig. 7.** The mean squared displacement, $\langle d^2 \rangle$, of individual cells is plotted versus the time interval, *t*, over which the displacements occur. The data points represent the pooled average of squared displacements over overlapping time intervals, taken from the tracks of 25 cells observed for 24 hours, in the absence (solid circles) or presence (open triangles) of 10 ng/ml TGF-β, 1 mM β-D-xyloside (open squares) or TGF-β and β-D-xyloside (solid diamonds), see Fig. 6. The lines are the nonlinear least squares regression fit of equation (1) to these data for each condition. The regression analysis estimates the motility coefficient, μ, for each condition. Note that equation (1) predicts that $\langle d^2 \rangle$ is asymptotically linear with *t*, with slope equal to 4μ. Thus, the plot provides an empirical comparison between the conditions, i.e. larger asymptotic slopes correspond to greater values of μ, and therefore a greater efficiency of cell migration.

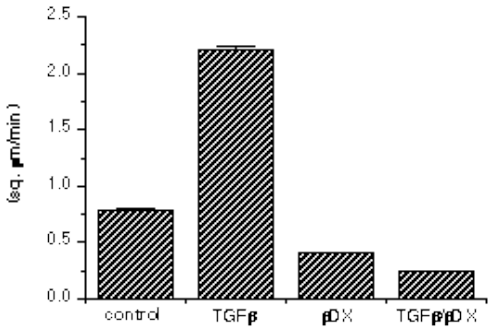


**Fig. 8.** β-D-xyloside inhibits TGF-β-stimulated melanoma cell migration on the surface of collagen gels, as quantified by the motility coefficient, μ, which reflects the efficiency of cell migration, i.e. the ability of a cell to move a greater total distance (on average) over a given period of time. This parameter was estimated from a nonlinear least squares regression fit of equation (1) to data for mean squared displacement, $\langle d^2 \rangle$, versus time, *t* (see Fig. 7, and Materials and Methods). Error bars represent standard errors in the parameter estimates obtained from the regression analysis.

TGF-β enhances cell migration. Conversely, μ is decreased following β-D-xyloside-treatment, regardless of the presence of TGF-β, corresponding to an inhibition of cell migration on the collagen gel. These results confirm that CSPG is required for efficient cell migration and suggest that the enhanced migration observed for TGF-β-treated cells requires CSPG. Note further that there is a direct correlation between the treatment effects on cell migration and the invasive potential of a cell population (Fig. 5).

## DISCUSSION

Our studies demonstrate that TGF-β enhances the migration and invasive behavior of mouse melanoma cells on collagen substrata, consistent with the results from other cell types (Mooradian et al., 1992). We have also found that TGF-β upregulates the expression of mouse melanoma CD44-CSPG. Cell surface CD44-CSPG has previously been shown to play a role in melanoma cell migratory and invasive behavior on collagen matrices (Faassen et al., 1992). In our current studies, removal of cell surface CSPG inhibited TGF-β-enhanced melanoma cell migration and invasive behavior, indicating that CSPG is required for this TGF-β-mediated stimulation.

TGF-β-upregulation of the expression of mouse melanoma cell surface CD44-CSPG occurs specifically through the stimulation of CS synthesis, by increasing the length of CS chains on CD44-CSPG core proteins. This result is consistent with the effect of TGF-β on syndecan in mouse mammary epithelial cells (Rapraeger and Rasmussen, 1989; Rasmussen, 1988) and arterial smooth muscle cell-associated proteoglycans (Chen et al., 1987), where the amount of CS per core protein is dramatically increased. In contrast TGF-β has been shown to modify extracellular matrix PG not only through an increase in total mass of the GAG chains, but also in the expression of the core proteins (Bassols and Massague, 1988; Massague, 1990). However, we did not detect any alteration in the synthesis of mouse melanoma CD44-CSPG core protein in the presence of TGF-β. While cell surface CSPG expression was increased in mouse melanoma cells in response to TGF-β, cell surface CSPG was not detectable in melanoma cells cultured in the presence of β-D-xyloside (Faassen et al., 1992), which blocks CS addition to the core protein (Schwartz, 1977). Consistent with this, β-D-xyloside inhibited TGF-β-stimulated cell migration and invasive behavior on type I collagen gels, confirming our previous results that cell surface CSPG plays a fundamental role in mouse melanoma cell invasive behavior (Faassen et al., 1992). The increased CS chain length detected on CD44-CSPG in TGF-β-treated cells could contribute to increased cell migration and invasive behavior, since the CS portion of CD44-CSPG was shown to be a critical component in mediating cell motility (Faassen et al., 1992).

We determined that TGF-β-treated cells have an increased ability to migrate on the surface of the collagen gel, while removal of CSPG diminishes the migration capacity of melanoma cells, regardless of the presence of TGF-β. The morphology of melanoma cells on the collagen gels in the various conditions is consistent with the migratory ability of the cells. We observed that TGF-β-treated cells tend to establish a more elongated morphology (see Fig. 6a), characteristic of a motile cell (Trinkaus, 1985; Soll et al., 1988), while β-D-xyloside treatment resulted in a more rounded cell shape. While the ability of the cell to establish a polarized morphology may be influenced by the degree of collagen fibril alignment in the assay, the failure of cells to assume a polarized morphology on aligned fibrils in the presence of β-D-xyloside suggests that the cell morphology is a result of the treatment itself and not of differences in fibril organization. Similarly, changes in the morphology of human pulmonary adenocarcinoma cells on collagen gels in the presence of TGF-β include the appearance of numerous long psuedopodia, consistent with a change in the motile behavior of these cells (Mooradian et al., 1992).

Although the removal of mouse melanoma CSPG by β-D-xyloside resulted in a more circular cell shape (i.e. less polarized), the area of cell spreading (i.e. projected area) on the collagen gel was not significantly different between cell treatments (data not shown). This suggests that CSPG mediates subtle effects on the cells ability to modulate and reorganize its cytoskeleton and establish a polarized morphology. By video microscopy we noted that the β-D-xyloside treated cells exhibited little directional persistence in movement, and were unable to form stable leading lamellae. In the absence of cell surface CSPG, the melanoma cells may be less able to form and/or maintain dominant lamellae, and thus possess a diminished capacity to migrate efficiently on the collagen gel.

Although some investigators have suggested that CSPG disrupts cell adhesion and thus may facilitate cell detachment and motility (Culp et al., 1986; Ruoslahti, 1988), other studies argue that this may not be a property attributable to all CSPG. Much of the data describing a de-adhesive function for CSPG relied on assays using CSPG from a variety of sources to compete for cell adhesion to various ECM ligands. From these studies it was suggested that CSPG may interfere with cell attachment 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; Gallagher, 1989). In contrast, other studies have identified a cell surface CSPG on microspikes of human melanoma cells (Garrigues et al., 1986), consistent with recent evidence implicating cell surface CSPG in mediating early recognition events in melanoma cell adhesion on defined fragments and synthetic peptides from fibronectin (Iida et al., 1992). Furthermore, a CS/HS-containing CD44 expressed by human keratinocytes has been localized to microspikes in cells adherent to collagen substrates (Brown et al., 1991). Although earlier studies demonstrated a redistribution of CD44 to the trailing edge of a moving fibroblast (Jacobsen et al., 1984), modification of CD44 with GAG may greatly alter the functional properties of this cell surface receptor. While we cannot rule out the possibility that CD44-CSPG may weaken cell adhesion at the trailing edge of a moving cell, our current results are consistent with a role for CD44-CSPG in mediating initial cell-ECM contacts required for forming and/or maintaining dominant leading lamellae. Our previous data indicating that

purified mouse melanoma CD44-CSPG binds to type I collagen (Faassen et al., 1992) supports the notion that CD44-CSPG mediates early interactions with the ECM during cell movement.

The activity of CSPG may be dependent upon the ability of a membrane bound core protein to transmit information to the cell interior (Yamagata et al., 1989; Gallagher, 1989; Hardingham and Fosang, 1992; Iida et al., 1992). Membrane PGs have been proposed to activate protein kinase C, and thus stimulate intracellular messenger systems (Woods and Couchman, 1992). Other studies have suggested that the cytoplasmic tails of transmembrane PG core proteins, such as syndecan, may provide phosphorylation sites for protein kinases or may form sites for interaction with cytoskeletal elements (Hardingham and Fosang, 1992). Importantly, the phosphorylation of serine residues on the cytoplasmic tail of CD44 enhances its binding affinity with the cytoskeletal component ankyrin (Kalomiris and Bourguignon, 1988; Carter and Wayner, 1988). By mediating early recognition events, mouse melanoma CD44-CSPG may participate in signalling events that modulate the cellular machinery involved in locomotion.

While cell surface CSPG is clearly implicated in TGF-β-stimulated melanoma cell migration, the migration efficiency of melanoma cells may be enhanced in the presence of TGF-β by additional mechanisms which act in combination with CD44-CSPG. In addition to augmenting proteoglycan synthesis, TGF-β induces a multiplicity of biological responses, some of which may influence cell adhesion and migration on ECM components (Massague, 1990). TGF-β has been shown to alter cell adhesion by modifying the repertoire of cell surface integrin receptors (Ignotz and Massague, 1987). Additionally, TGF-β has been implicated in phospholipase C-mediated hydrolysis of membrane phospholipid (Diaz-Meco et al., 1992), which could serve to enhance the turnover of phospholipid-linked cell adhesion receptors, such as MPIHP-63, the cell surface heparan sulfate PG expressed by these mouse melanoma cells (Drake et al., 1992). TGF-β has also been shown to activate phosphatidylinositol turnover and modulate second messenger generation (Muldoon, 1988), critical for the generation of a motile response (reviewed by Lester and McCarthy, 1992). Defining the relationship between these TGF-β-mediated effects and levels of CSPG expression may help to elucidate the molecular mechanism by which mouse melanoma CD44-CSPG influences cell migration and invasiveness.

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