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Versican Enhances Locomotion of Astrocytoma Cells and Reduces Cell Adhesion through Its G1 Domain

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Abstract. Versican is a large extracellular proteoglycan and is expressed in a variety of tissues including the central nervous system. A malignant astrocytoma cell line U87 with high motility expressed a higher level of versican than another malignant astrocytoma cell line U343 with lower motility. We observed that the U87 cells were less adherent to tissue culture plates than the U343 cells. To investigate the role of versican in astrocytoma cell migration, we generated recombinant products of a mini-versican construct expressed in COS-7 cells. We found that the mini-versican products enhanced astrocytoma cell migration. Furthermore, enhanced migration was promoted by the G1 domain but not the G3 domain of versican. We introduced culture medium containing products of the mini-versican, the G1, and the G3 constructs separately into the astrocytoma cell lines U87 and U343. The mini-versican and the G1 construct, but not the G3 construct, were shown to reduce astrocytoma cell adhesion. The present data suggest that versican exerts its effect on astrocytoma cell migration and adhesion through the G1 domain.

Key Words: Adhesion; G1 domain; Hyaluronan; Locomotion; Proteoglycan.

INTRODUCTION

Astrocytoma is the most common tumor in the central nervous system (CNS) in all age groups and represents about 30% to 40% of all CNS tumors. Astrocytomas are divided into 4 grades based on the recent WHO Grading System (1), with the higher-grade tumors correlating with poorer outcome. Most astrocytomas have a propensity to invade adjacent brain tissue, white matter tracts, and subependymal and the subpial tissue, and this property is largely responsible for the poor outcome of patients afflicted with higher-grade tumors (2).

Versican, a chondroitin sulfate proteoglycan (CSPGs) located in the extracellular matrix (ECM), is known to be expressed in the human CNS (3). It is composed of a G1 domain, a G3 domain, and a large sequence also named CS domain between the G1 and G3 for the attachment of chondroitin sulfate chains (4–6). The length of the CS domain varies with alternative splicing. As a result, versican has at least 4 different isoforms (7, 8). Structurally, versican is similar to aggrecan, neurocan, and brevican in that each molecule contains the G1 domain and the G3 domain (9–11). The G1 domain is composed of an IgG-like motif and 2 tandem repeats, while the G3 domain contains 2 epidermal growth factor (EGF)-like motifs, a carbohydrate recognition domain (CRD), and a complement binding protein (CBP) motif. Like other chondroitin sulfate proteoglycans, versican has been reported to inhibit the adhesion of cells to substrata. Versican may also repress focal contact formation between cells and inhibit cell adhesion in fibroblast cultures (12). It has been reported previously that versican interferes with the attachment of cells to various extracellular matrix components such as collagen I, fibronectin, and laminin (13), and also inhibits intercellular adhesion of normal cells (14, 15) as well as malignant tumor cells (16). Nara et al (17) examined the immunolocalization of versican in breast tumors, including infiltrating ductal carcinoma, benign tumors, and fibrocystic diseases. Versican was found in the proliferating hyaluronic acid-rich interstitial tissues, at the peripheral invasive areas of the infiltrative carcinoma, and in the vascular and perivascular elastic tissues associated with tumor invasion. Of particular interest was the observation that in the peripheral areas of infiltrating ductal carcinoma there was intense versican expression in the mesenchymal tissues between the invasive clumps of carcinoma cells and the surrounding hyaluronic-acid rich tissue. This study suggests the "specific stroma" at the invasive front of infiltrating ductal carcinoma is rich in versican and underscores the importance of this anti-adhesive molecule in tumor invasion (17). This finding is consistent with that found in the skin where versican is localized at the proliferating zone of the epidermis (18). During development, versican is also expressed at the interphase between actively mobile migrating neural cells of the embryonic tissue and the surrounding mesenchyme (19).

Using malignant human astrocytoma cell lines, we conducted experiments to determine the specific domain in the versican molecule that could influence the invasive property of astrocytoma. We have demonstrated that a mini-versican and the G1 domain of versican not only reduced cell adhesion, but also enhanced glioma cell migration activities involved in tumor invasion.

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MATERIALS AND METHODS

Immunostaining of Endogenous Versican in Astrocytoma Cell Lines

The human malignant astrocytoma cell line U87 was obtained from the American Type Culture Collection (Rockville, MD) and U343 from Brain Tumor Research Center, University of California (San Francisco). The cells were maintained in Dulbecco's modified eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) at 37° C in 5% CO_2. The cells were cultured on glass slips in Costar's Plastic Leighon Tube with cap and plastic cover slip and grown at 37°C for 2 days. The cells were fixed with paraformaldehyde and probed with a primary polyclonal antibody against versican that was raised and purified by us previously (20). The cells were then stained with horseradish peroxidase-conjugated goat-anti-rabbit IgG secondary antibody and colored with 3-amino-9-ethylcarbazole (AEC). Each cell line immunostained with versican was reviewed under the light microscope, and the field with the highest number of immunostained cells was selected for assessment.

Gene Construction and Expression

A recombinant mini-versican gene was constructed by ligating the G1 domain, a fragment of the CS sequence, and the G3 domain, which were cloned separately as described earlier (20). This construct has already been shown to possess characteristic proteoglycan properties, namely, attachment of glycosaminoglycan chains (GAGs) and secretion of the gene product. For this study, a G1 construct was generated from the recombinant versican using PCR. 5′-AAA AAG CTT GCC GCC ACC ATG GTG TTA AAC ATA AAA AGC ATC and 5′-AAA GCA TGC TTC GTA GCA GTA GGC ATC were used as primers. The resulting G1 construct was transiently transfected into COS-7 cells. Analysis of the gene product demonstrated that the G1 construct was expressed, but not secreted (data not shown). This means that the signal peptide of versican was not able to bring the G1 out of the cells. We have demonstrated previously that the signal peptide of link protein is sufficient for secretion of G1 or G3 of aggrecan to the matrix (21). Thus, the signal peptide of link protein was added to the G1 construct. To do this, 5′-AAA GAA TTC GCC GCC ACC ATG GCA AGT CTA CTC TTT CTG and 5′-AAA GGA TCC CTC GAG AGG CAG TGT GAC GTT GCC were used as primers in a PCR reaction to generate a DNA sequence containing the signal peptide of link protein. 5′-AAA GGA TCC ACT CTA CTA CTA GTG and 5′-AAA GCA TGC TTC GTA GCA GTA GGC ATC were used to generate the G1 domain without the signal peptide of versican. The PCR products were purified using agarose gel electrophoresis and a Prep-A-Gene DNA purification kit. The PCR product of signal peptide was digested with EcoRI and BamHI, while the G1 domain was digested with BamHI and SphI. These 2 cDNA fragments were linked together and inserted into EcoRI- and SphI-digested pcDNA1. We have provided a detailed description of the constructs in an earlier report (20). The leading peptide of link protein contains an epitope recognized by a monoclonal antibody 4B6 (22).

In generating the G3 construct, the leading peptide of link protein (23) was joined with the versican G3 domain in order to allow secretion of the G3 gene product. The leading peptide of link protein was amplified with the primers 5′-AAA GAA TTC GCC GCC ACC ATG GCA AGT CTA CTC TTT CTG and 5′-AAA CTC GAG AGG CAG TGT GAC GTT GCC in a PCR reaction. The PCR product was purified and digested with EcoRI and XhoI. The G3 domain was derived from the mini-versican construct using the restriction enzymes XhoI and SphI. The G3 fragment and link protein leading peptide were ligated into the pcDNA1 vector digested with EcoRI and SphI.

The recombinant genes constructed in pcDNA1 were transfected transiently into COS-7 cells (American Type Culture Collection) using Lipofectin (GIBCO) as originally described by Felgner et al (24). The growth medium and cells were harvested separately after 3 days of transfection and analyzed with western blot assays.

Strategy for insertion of the G1 fragment into pQE30 (Qiagen Inc., Chatsworth, CA, Cat. No. 32149) is shown in Figure 2A. pQE30, a bacterial expression vector, contains an epitope (MRGSHHHHHH) at its amino terminus recognized by a monoclonal antibody anti-^{MRGS}His. The 6 histidines in this epitope bind Ni and allow purification of fusion proteins on a Ni-NTA affinity column. The epitope is followed by multiple cloning sites. The G1 construct generated above was flanked with BamHI and SphI and inserted into BamHI- and SphI-digested pQE30 (Qiagen). The resulting construct contained an N-terminal ^{MRGS}His tag and was expressed in E. coli strain M15. Gene products produced by bacteria were purified on a Ni-NTA affinity column (Qiagen, Cat. No. 30230) according to the manufacturer's instructions.

To allow expression in pQE30, the G3 fragment was amplified with 2 primers, 5′-AAA GGA TCC GGA CAG GAT CCA TGC AAA-3′ and 5′-AAA GCA TGC GCG CCT TGA GTC CTG CCA-3′ in a PCR reaction, as above, and ligated into the bacterial expression vector pQE30. The resulting construct contained an N-terminal ^{MRGS}His tag and was expressed and purified as described above.

Western Blot

Electrophoresis of recombinant proteoglycans was performed in SDS-PAGE-western blot assay. Cell lysate and growth medium that contained recombinant gene products were subjected to SDS-PAGE electrophoresis. A 4% separating gel was used for the mini-versican product and a 12% separating gel for the G1 and G3 domains. The

buffer system is 1× TG (Tris-glycine buffer, Amresco product) containing 1% SDS. After electrophoresis, proteins were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1× TG buffer containing 20% methanol. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 8% nonfat dry milk powder (TBSTM) for 1 h at room temperature, and then incubated at 4°C overnight with anti-versican polyclonal antibody (1:1,000 dilution) in TBSTM. The membranes were washed with TBST (3 × 30 min) and then incubated for 1 hour with goat anti-rabbit antibody conjugated to horseradish peroxidase in TBSTM. After washing as above, the bound antibody was visualized with an ECL kit according to the manufacturer's instructions (Amersham).

Electrophoresis of endogenous versican was performed in agarose gel (agarose-western blot assay) using glioma cell lysate. The agarose gel was 4 cm in height containing 1.5% agarose in a barbital buffer (0.124 M Tris-HCl, 27-mM barbituric acid, 1 mM EDTA, pH 8.7). This agarose gel was made on top of a 1-cm conventional 10% polyacrylamide gel. The polyacrylamide gel was used only to seal the bottom of the casting stand and acted as a base for the agarose separating gel. The barbital buffer was also used as a running buffer, and the electrophoresis took place at 50 v for 5 h at room temperature. Molecules up to 2 million Da in size (such as Blue Dextran 2000) are able to enter the agarose gel. Crude extract of brain tissues and glioma cells of equal protein concentrations were analyzed in the gel. To allow transfer of such large molecules onto the nitrocellulose membrane, the blotting took place in TG buffer at 20 v overnight at 4°C. The rest of the procedure was similar to the western blot protocol described above. The primary antibody was rabbit polyclonal antibody against versican and the secondary antibody was horseradish peroxidase-conjugated goat-anti-rabbit IgG.

Purification of Recombinant Products

To study the role of the mini-versican in cell migration, the mini-versican products were purified. Briefly, COS-7 cells were transiently transfected with the ^{MRGS}·His-tagged mini-versican constructed. Four days after transfection, growth medium and cell lysate were harvested with lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0). This buffer was also used to equilibrate a Ni-NTA column. The samples were applied to the Ni-NTA columns, and the columns were washed extensively with washing buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3). The mini-versican products were eluted with elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5). The eluted product was dialyzed extensively against PBS and analyzed in western blot and silver staining to confirm that the mini-versican had been purified.

To study the effect of G1 on glioma cell adhesion, G1 products were purified from the G1-transfected E. coli strain M15. Briefly, cell lysate from G1-transformed bacteria, was suspended in equilibration buffer. The samples were applied to the Ni-NTA columns and the columns were washed extensively with washing buffer. The G1 peptides were eluted with elution. The purified products were dialyzed against PBS, analyzed on SDS-PAGE, and visualized with Commassie blue dye staining.

Astrocytoma Migration Assay

To evaluate astrocytoma cell migration in response to versican expression, glioma cells were cultured in tissue culture plates at a density of 10^4 cells/ml. After overnight incubation, the cells were wounded by removal of half the monolayer with a cell scraper. The edge of the wounding was marked with a permanent ink pen. The cells were incubated with different reagents to test their effects on cell migration. These wounded cells were incubated in a tissue culture incubator for 3 days. The cells that migrate from the edge were examined under light microscopy and photographed. Each photograph was printed on photographic paper. The magnification used in the microscopy was ×40. When the pictures were printed, the magnification was ×4. Therefore, the magnification of the pictures for cell migration is ×160. To calculate the distance of cell migration, the distance between each cell to the wounding edge was measured manually. Fifty cells were randomly selected for the measuring. The distance was expressed as mm that equal the distance seen under the microscopy (×40). It also equals the distance seen in the contact sheet of the negative film.

Astrocytoma Adhesion Assay

Astrocytoma cells were seeded in 24-well plates at a concentration of 2 × 10^4 cells/well in 0.5 ml DMEM containing 5% FBS and allowed to grow to 90% confluence. Cell adhesion was determined at different concentrations of EDTA prepared in Hank's balanced salt solution. Growth medium was removed and cells were incubated with 0.5 ml of EDTA solution for 5 min with shaking at a speed of 50 rpm in a platform shaker at room temperature. The EDTA solution was removed and cells were washed with PBS. Cells remaining adherent to the plates were washed gently with PBS once to remove the cells, which sit on but do not spread, from the plates. The high adhesion cells that remained adherent to the plates after the wash were incubated with 10 mM EDTA (0.5 ml) for 10 min for complete cell detachment. Cells in each well were agitated with a micropipette to obtain a single-cell suspension. The cell number in each well was determined using a cytometer. Cell adhesion was expressed as: Cell adhesion (%) = T/C, where T is the number of cells that remain adherent to the plates in each

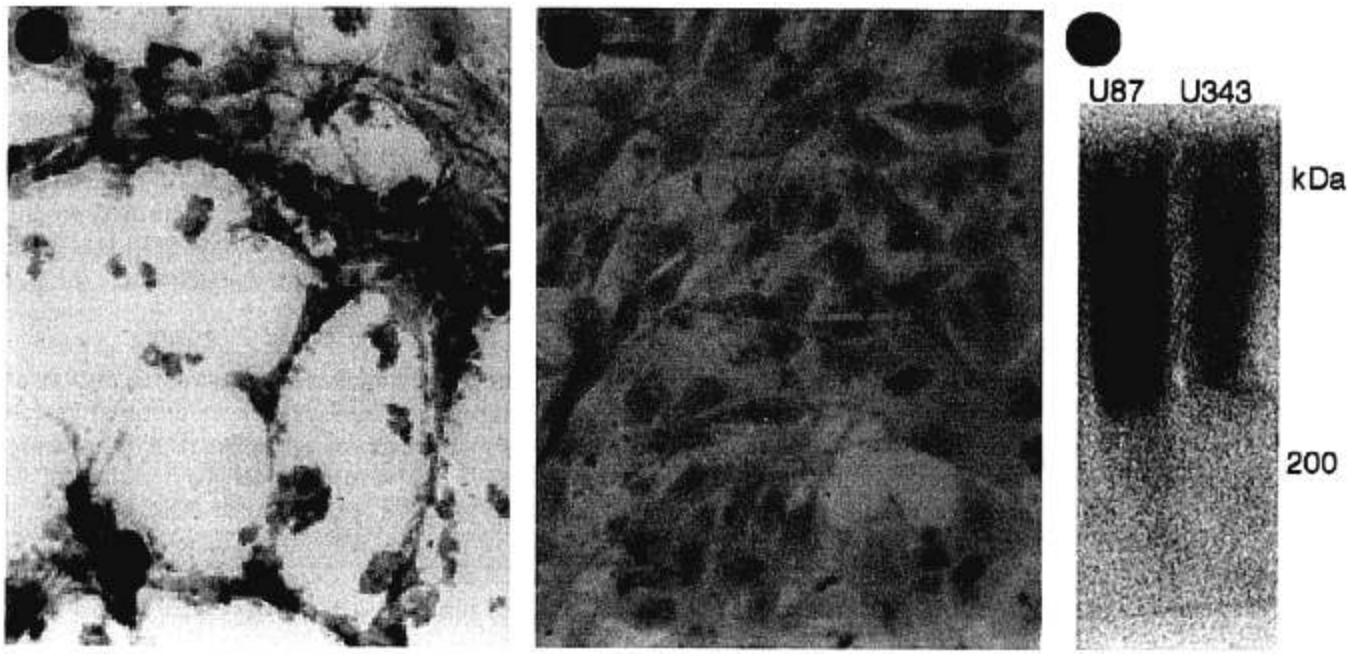

Fig. 1. Endogenous versican expression in astrocytoma cell lines. Astrocytoma cell lines U87 (A) and U343 (B) are probed with a polyclonal antibody against versican. The photomicrographs have a magnification of ×450. U87 has a more intense versican-immunoreactivity than U343. Cell lysate is harvested and equal amount of proteins are analyzed for versican expression in Western blot. U87 cells produce higher level of versican than do the U343 cells (C).

well and C is the total cell number without EDTA-treatment (control). Each treatment had 1 control and was done in quadruplicate.

RESULTS

Versican Promotes Astrocytoma Cell Migration

It has been reported that versican is expressed in brain tumors (3). To investigate the functions of versican in mediating cellular activities, we analyzed the expression of versican in 2 human malignant astrocytoma tumor cell lines, U87 and U343. We observed that the U87 (Fig. 1A) expressed higher levels of versican than the U343 (Fig. 1B). Versican appears to bind to the glioma cell surface (Fig. 1A). Otherwise, versican was secreted to the culture medium. This was confirmed by Western blot probed with the polyclonal antibody to versican (Fig. 1C). This result is consistent with our previous studies on the expression of endogenous versican using the same antibody to versican (20).

In cell migration assay, U87 cell line (Fig. 2A) had a higher rate of locomotion than U343 cell line (Fig. 2B), and the difference is statistically significant (Fig. 2C). In the next stage, we investigated whether versican expression was involved in cell migration. It is known that versican binds to hyaluronan (25), a molecule that possesses the properties of stimulating cell migration by binding to hyaluronan-binding receptor and by destabilizing cell adhesion. Our plan was to determine if versican also plays similar roles in cell migration and adhesion. A recombinant mini-versican (Fig. 3A) was transiently expressed in COS-7 cells. Cell lysate and growth medium were prepared for analysis of versican expression in a western blot assay. When the recombinant gene was expressed, the product in the growth medium showed a diffuse band stained by a monoclonal antibody 4B6 that recognized an epitope in the signal peptide of the mini-versican construct, characteristic of proteoglycans (Fig. 3B).

In order to determine whether the mini-versican gene product acts at the extracellular matrix and produces any effect on cell migration, we introduced exogenously the growth medium containing the mini-versican gene product into an astrocytoma cell line U87. Growth medium from vector-transfected cells was used as a control. Cell migration was monitored using a microscope and photographed. The culture medium from the mini-versican-transfected COS-7 cells had a greater ability to enhance astrocytoma cell migration (Fig. 3C) than that from vector-transfected cells (Fig. 3D). The migratory distance of 50 cells from the wounding edge was measured and calculated. The result showed that the difference between the culture medium from the mini-versican- and vector-transfected cells was significant (Fig. 3E). The mini-versican products were purified with a Ni-NTA affinity column from COS-7 cells transfected with the mini-versican construct. Vector-transfected cells were used as controls as described in the Materials and Methods section. The

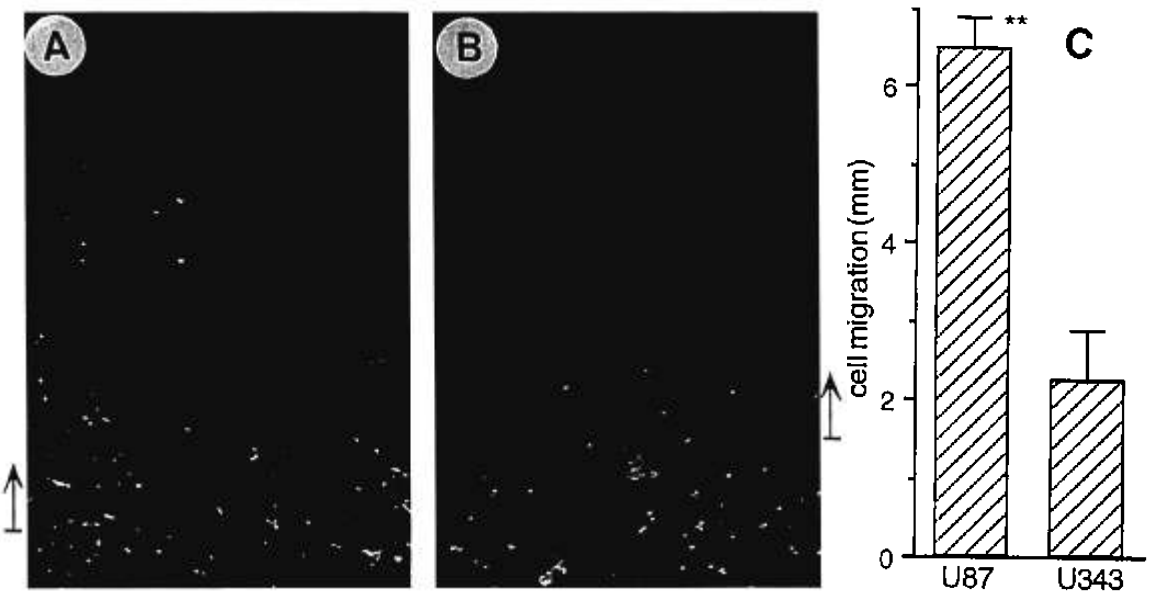

Fig. 2. Cell migration assays in astrocytoma cell lines. Astrocytoma cell migration is tested in 6-well tissue culture plates. After overnight culture, half of the cultures from each well are removed with cell scrapers. The cultures are maintained in an incubator. Migration of the astrocytoma cells are photographed. More cells of the U87 glioma cells migrate away from the injured line (A) than the U343 cells (B). The migration photomicrographs have a magnification of ×160. The arrows indicate the start and direction of cell migration. Measurement of the distance of migration indicates that the levels of migration is significantly higher in U87 cells than in U343 cells (C, n = 50; ** $p < 0.01$).

result showed that purified mini-versican products enhanced glioma cell migration significantly compared with the controls (Fig. 3F). We routinely purify the mini-versican using a Ni-NTA affinity column. We have previously analyzed the yield and purity of the products (20).

Aggrecan G1 domain binds hyaluronan. The versican G1 domain is structurally similar to the aggrecan G1 domain and may bind hyaluronan resulting in enhancing cell migration. To investigate the possibility that versican exerts such effect through the G1 domain, we expressed versican G1 construct and the G3 construct in COS-7 cells. Expression of these constructs were confirmed by western blot (Fig. 4A). Growth medium from the G1-, G3-, and vector-transfected cells were introduced into the wounded U87 cell cultures. The culture medium from the G1-transfected cells enhanced glioma cell migration (Fig. 4B) as compared with those from the G3- (Fig. 4C) or vector-transfected cells (Fig. 4D). Measuring of the migration distance from the cells to the wounding edge indicated that the differences were significant (Fig. 4E).

Versican Reduces Astrocytoma Cell Adhesion

To test if versican plays a role in astrocytoma cell adhesion while it enhances cell migration, 2 malignant astrocytoma cell lines, U87 and U343, were cultured in 24-well tissue culture plates and tested for their ability to adhere to the plates. The U87 cell line, which expressed higher level of versican, had lower activity of attachment than the U343 cell line, which expresses lower level of versican (Fig. 5A). To confirm the function of versican in astrocytoma cell adhesion, the U87 cell line was cultured in the same plates as above and culture medium from the mini-versican- or vector-transfected COS-7 cells was introduced into the cultures. Astrocytoma cell adhesion was tested as above. The culture medium from the mini-versican-transfected cells reduced the adhesion of the U87 cells to the culture plates (Fig. 5B).

We have previously demonstrated that aggrecan mediates cell-substratum interaction through the binding of its G1 domain to hyaluronan and link protein (26). The versican G1 domain is structurally similar to the aggrecan G1 domain. To investigate the possibility that versican reduces glioma cell adhesion through the G1 domain, we expressed the versican G1 domain and G3 domain in COS-7 cells. After gene expression, the culture medium was collected and introduced into the glioma cell line U87 to test cell adhesion. The results confirmed that the addition of exogenous G1 gene product, but not the G3 gene product, decreased cell-substratum interaction (Fig. 6A).

We have previously demonstrated the versican G3 domain expressed by E. coli strain M15 was able to enhance cell proliferation although the efficiency was far lower than a G3 construct expressed by COS-7 cells (20). We tested the effects of the versican G1 domain expressed by E. coli strain M15 on glioma cell adhesion using the same protocol for product purification (20). The controls were G3 and vector transfections. We demonstrated that

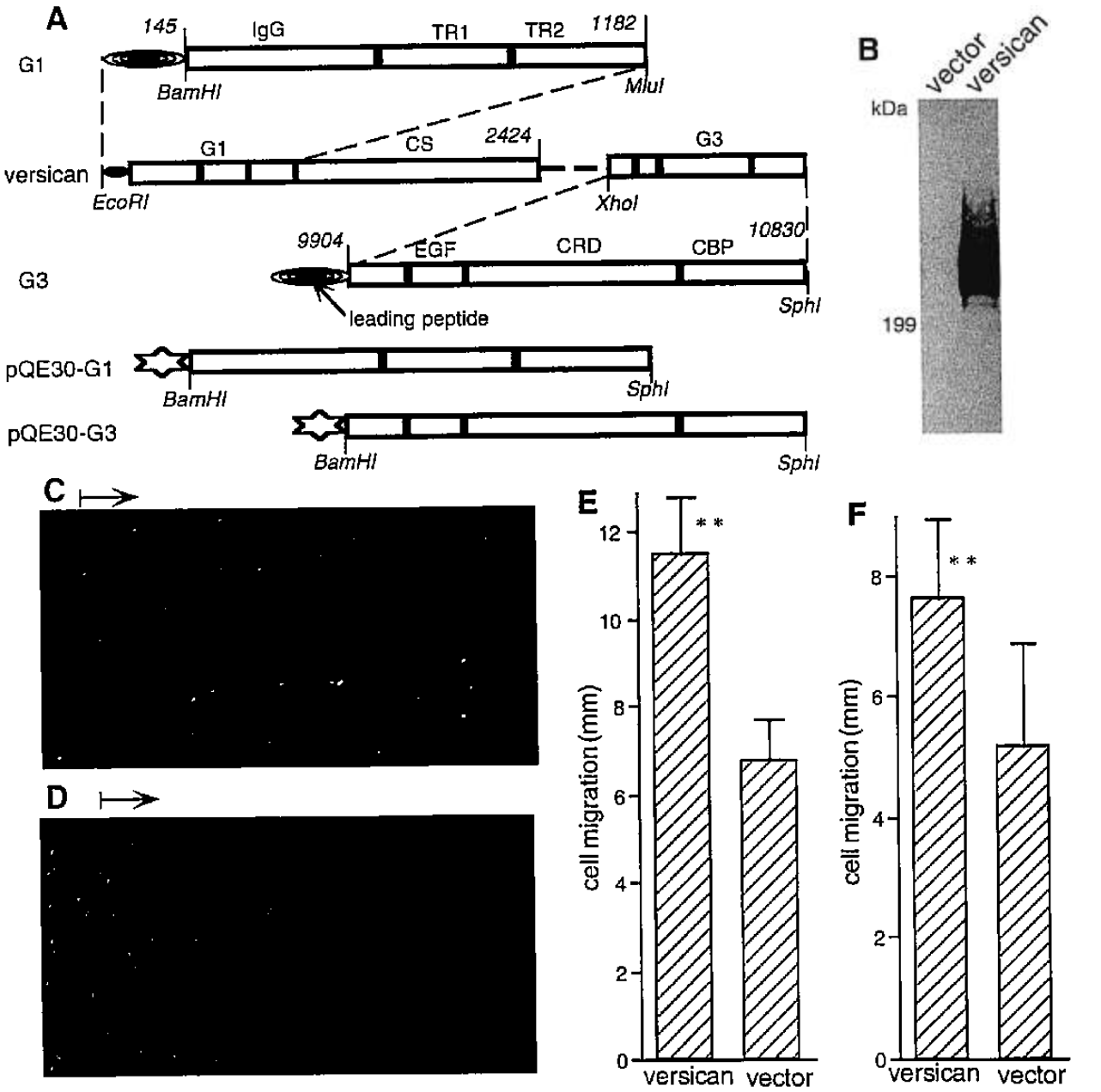

Fig. 3. Addition of exogenous growth medium containing the versican mini-gene product enhances astrocytoma cell migration. Astrocytoma cell line U87 is seeded in 6-well tissue culture plates and the cultures are wounded as above. Growth medium collected from versican- or the control vector-transfected COS-7 cells is mixed in a 1:1 ratio with standard medium, and the mixture is introduced into the astrocytoma cultures (2 ml/well). The recombinant versican construct is shown in the figure (A), which also includes a number of constructs used in this study. Expression of the recombinant mini-versican is analyzed with Western blot (B). The cultures are maintained in an incubator. Migration of the astrocytoma cells is photographed. More cells migrate away from the injured line when incubated with medium containing the mini-versican gene product (C) than with medium from the vector-transfected cells (D). The arrows indicate the start and direction of cell migration (×160 magnification). The migration distance is measured. The mini-versican enhances cell migration significantly as compared with the control vector (E, $n = 50$; ** $p < 0.01$). The mini-versican product is purified and the purified products enhance cell migration significantly compared with control (F, $n = 50$; ** $p < 0.01$).

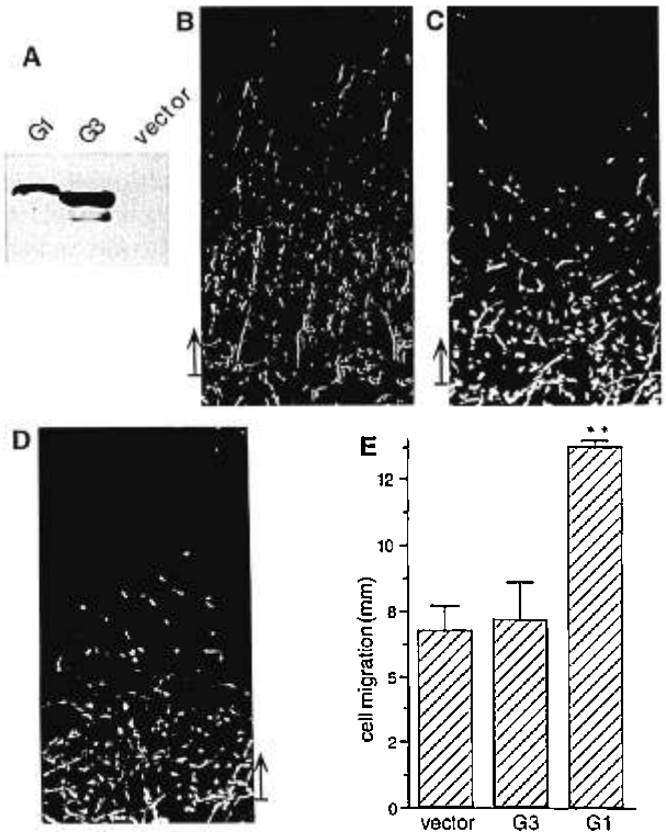

Fig. 4. Versican G1 domain, but not G3 domain promotes astrocytoma cell migration. U87 cultures are wounded as above. Growth medium collected from the mini-versican-, G1- or G3-transfected COS-7 cells is mixed in a 1:1 ratio with standard medium, and the mixture is introduced into the wounded cultures (2 ml/well). Expression of both constructs is analyzed with Western blot (A). Cell migration is tested and photographed. More cells migrate away from the injured line when incubated with medium containing G1 gene product (B) than with medium from the G3- (C) or the vector-transfected cells (D). The arrows indicate the start and direction of cell migration (×160 magnification). The migration distance is measured (E). Growth medium from G1-transfected cells enhances glioma migration significantly ($n = 50$; ** $p < 0.01$).

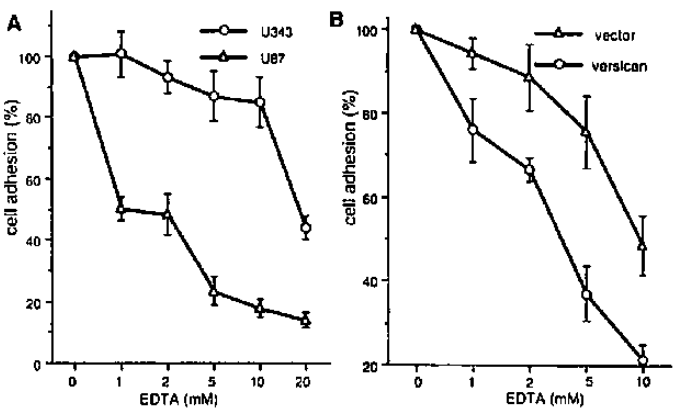

Fig. 5. Versican reduced astrocytoma cell adhesion. A) Astrocytoma cell lines U87 and U343 are seeded in 24-well tissue culture plates and maintained in a tissue culture incubator over night. Cell adhesion is tested as described in the Methods section. U87 cells have lower adherent ability than U343. B) U87 cells are cultured as above. Growth medium collected from versican- or vector-transfected COS-7 cells is mixed in a 1:1 ratio with standard medium and the mixture is introduced into the astrocytoma cultures (0.5 ml/well). The cultures are maintained overnight, and cell adhesion is assayed with different concentrations of EDTA as indicated in the figure. Growth medium is removed and the cells are incubated with EDTA solution (0.5 ml/well) for 5 min at room temperature to remove low adherent cells from the plates. Cell adhesion is calculated as described in the Materials and Methods section. Growth medium from the mini-versican-transfected cells has higher ability to reduce cell adhesion.

the purified G1 product reduced glioma cell adhesion compared with control (Fig. 6B).

DISCUSSION

The large chondroitin sulfate proteoglycan versican is highly expressed in CNS (3) where it associates with a number of molecules in the ECM such as hyaluronan (25), tenascin (27), and fibronectin (28). In the CNS, tenascin binds to the C-type lectin-like motif. Aggregates composed of hyaluronan, versican, and tenascin are potential candidates in mediating the interactions of cells within specific tissue structures and the ECM.

Several lines of evidence indicate that versican possesses anti-adhesive properties. First, human MG63 osteosarcoma cell line transfected with versican antisense RNA showed the suppression of malignant cell-adhesive phenotype, which correlated well with the reduction of versican biosynthesis (15). The podosomes, which are usually present in migratory cells, in this instance were replaced by focal adhesion-like structures with microfilaments terminating in these structures. This type of cytoskeletal organization resembles that of non-neoplastic fibroblasts grown in fibronectin-rich monolayer culture. Second, it has been shown that a substrate composed of versican and fibronectin does not support attachment of L929 cells as well as a substrate composed of fibronectin alone. Lastly, in various fibroblast cultures, versican was found to be abundant in the subcellular space, but was selectively excluded from focal contacts where vinculin, integrins, and fibronectin were localized (12). The distributions of hyaluronan, CD44, and tenascin were similar to versican, suggesting that these molecules may not be involved in focal contact. Interestingly, this same study had demonstrated the tracks left by migratory fibroblasts on the culture plates exhibited versican immunoreactivity (29). Previous experiments have demonstrated that chick mesenchymal cells migrating in a collagen substrate deposited a material rich in CSPGs in their track (30). This deposited material is thought to contain versican, as it is a major ECM component of CSPGs secreted by mesenchymal cells.

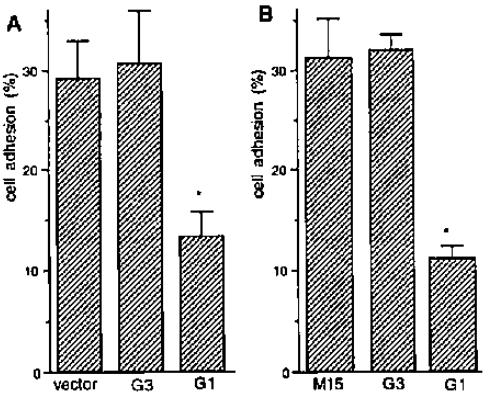

Fig. 6. Astrocytoma cell adhesion reduced by the G1 domain, but not the G3 domain. A) Astrocytoma cells (U87) are seeded in 24-well tissue culture plates. Growth medium from the G1-, G3-, or the control vector-transfected COS-7 cells is mixed with onefold standard medium and introduced into the cultures followed by overnight incubation. Cell adhesion is assayed as described above using 5 mM EDTA. The level of reduced adhesion is significantly greater in cells treated with medium from G1-transfected cells than from vector- and G3-transfected cells ($n = 4$; * $p < 0.05$). B) Products from G1- or G3-transformed bacteria are purified using a Ni-NTA affinity column. Vector-transformed bacteria are used as controls in the purification. The elutes from the treatments, G1-, G3-, and vector-transformation are dialyzed extensively against PBS and incubated with glioma cultures. The G1 products reduces cell adhesion significantly compared with the G3 and vector controls ($n = 4$; * $p < 0.05$).

Using molecular biology techniques, we demonstrated that a versican mini-gene enhanced astrocytoma cell migration, which occurred through the G1 domain of versican. Because cells with higher activity of migration had lower activity of adhesion, we suspected that versican enhanced cell migration through the reduction of cell adhesion. This has been shown in the astrocytoma cell lines U343 and U87, where the latter has a higher rate of migration but lower rate of adhesion, while the former shows the reverse. Interestingly, U87 cells were observed to express much higher level of versican compared with U343 cells. Indeed, we have demonstrated that exogenous versican could further reduce the adhesion of U87. Our experimental evidence specifically points to the G1 domain of versican as being responsible for the destabilizing anti-adhesive activity of versican. A construct consisting of G1 alone was sufficient to inhibit adhesion. On the other hand, the G3 construct of versican has no effect in enhancing astrocytoma cell migration and reducing cell adhesion. Our data presented in the report were performed on tissue culture plates without protein coating. Initially, we coated the tissue culture plates with different gene products such as the mini-versican and the G1 and G3 products, and tested the effects of the gene product on cell migration. After 3 days of incubation we were not able to obtain reproducible results each time. Although the coated mini-versican had a trend in enhancing cell migration, the results from G1- and G3-coating were not consistent. We explained that the globular proteins did not attach to the plates as well as the large chondroitin sulfate proteoglycan mini-versican. Additionally, the wounding procedure, in which we used a cell scraper to remove part of the culture from tissue culture plates, may release some coated proteins from the plates causing inconsistency of the results. Therefore, we have to perform the migration assays directly on tissue culture plates. For the sake of comparison, the cell adhesion assays were also performed directly on tissue culture plates.

Conversely, U343 cells have a lower rate of locomotion but higher capacity for adhesion. Exogenous versican, however, was not able to significantly influence the locomotive and adhesive properties of U343 cells (data not shown). Consequently, we had to perform all studies in U87 cells. The lack of response of U343 to versican certainly warrants further investigation, as this may shed some light on those intrinsic factors present in cells that could inhibit or hinder migration. At the moment, we can only suggest that U343 cells do not respond to versican in the same way that U87 cells do. The mechanism underlying this phenomenon is complex, and there is no direct method to test them.

The only other study on versican expression in brain tumors that we are aware of (Paulus et al) examined the expression of versican spliced variants at the RNA and protein levels in a sample of 40 tumors, including astrocytoma, oligodendroglioma, medulloblastoma, schwannoma, and meningioma (3). There was increased expression of versican in the endothelial cells of cerebral blood vessels in the glioma, but low versican expression in glioma ECM. The authors felt that the versican expression in the glioma blood vessels could explain the low incidence of systemic metastasis in primary glioma. It is difficult to reconcile the above findings with our series of experiments that indicate that the G1 domain is linked to the anti-adhesive properties of versican. One explanation is that the antibodies used in this study were monoclonal antibodies directed against the GAG attachment region (CS) of versican and not against the G1 domain.

The molecular mechanism by which G1 exerts its effects is unknown. Whatever the mechanism may be, it is likely that hyaluronan is involved. Hyaluronan is an important molecule found in the extracellular matrix. It has been shown to destabilize cell-cell and cell-matrix interactions, to enhance cell migration, and to stimulate cell proliferation (31–33). As previously suggested, the G1 domain of versican has a capacity to bind hyaluronan (25). Tissues from organisms during early stages of development and a number of tumors also contain high levels of both versican and hyaluronan. It has also been reported that chondroitin sulfate side chains play a critical

role in inhibiting cell adhesion (14, 15). But neither the core protein nor the side chains alone are sufficient; a native form, consisting of core protein and side chains, is required for total inhibition of cell adhesion. These suggest that versican-mediated destabilization of cell adhesion is regulated by at least 2 independent mechanisms; one involving chondroitin sulfate chains and the other involving the G1 domain. Our study indicates that the G1 domain plays a critical, though not exclusive, role in the destabilizing effect of versican on astrocytoma cells by reducing cell adhesion and increasing cell migration. We believe that the ability of astrocytoma cells to migrate is a significant factor in the invasiveness of astrocytoma, and understanding the underlying mechanism for invasion could have future therapeutic implications in the management of such tumors.

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