

Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins

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SUMMARY

Tenascin, an extracellular matrix protein, is expressed in human gliomas *in vitro* and *in vivo*. The distribution of tenascin at the invasive edge of these tumors, even surrounding solitary invading cells, suggests a role for this protein as a regulator of glioma cell migration. We tested whether purified tenascin, passively deposited on surfaces, influenced the adhesion or migration of a human glioma-derived cell line, SF-767. Adhesion of glioma cells to tenascin increased in a dose-dependent fashion up to a coating concentration of 10 µg/ml. Higher coating concentrations resulted in progressively fewer cells attaching. Cell adhesion could be blocked to basal levels using anti-β1 integrin antibodies. In contrast, when anti-αv antibodies were added to the medium of cells on tenascin, cell adhesion

was enhanced slightly. Using a microliter scale migration assay, we found that cell motility on tenascin was dose dependently stimulated at coating concentrations of 1 and 3 µg/ml, but migration was inhibited below levels of non-specific motility when tested at coating concentrations of 30 and 100 µg/ml. Migration on permissive concentrations of tenascin could be reversibly inhibited with anti-β1, while treatment with anti-αv antibodies increased migration rates. We conclude that SF-767 glioma cells express two separate integrin receptors that mediate contrasting adhesive and migratory responses to tenascin.

Key words: Astrocytoma, ECM, Integrin, Migration, Tenascin

INTRODUCTION

The tenascin (TN) family, which includes at least three members, TN-C, -X, and -R, is composed of multifunctional extracellular matrix glycoproteins expressed in a variety of tissues (Erickson, 1993; Koukoulis et al., 1991). In the developing and adult central nervous system (CNS), this protein appears in a spatiotemporally regulated manner and its presence is linked to processes such as cell adhesion (Bourdon and Ruoslahti, 1989), migration (Riou et al., 1990), neuronal outgrowth (Lochter et al., 1991), proliferation (End et al., 1992), differentiation and cell death, as well as wound healing and angiogenesis (Erickson and Bourdon, 1989). The distribution of TN in the developing nervous system implicates this protein as a regulator of neural crest cell migration (Bronner-Fraser, 1988). For example, colonization waves of neural crest cells in the developing cornea correlate with the expression and disappearance of TN in the route of migrating cells (Kaplony et al., 1991). However, Saga et al. (1992) showed that knockout mice, completely lacking TN-C expression, developed normally with no significant histological changes in various tissues including the nervous system (Saga et al., 1992). It remains unclear whether paralogue proteins like TN-R and TN-X duplicate the function of TN-C or whether the role of this protein in development is, in fact, minor.

TN is composed of several distinct domains containing epidermal growth factor-like (EGF) repeats, 6-16 fibronectin type III repeats (Tnfn) and a segment of great homology with the β and γ chain of fibrinogen (TNfbg) (Gulcher et al., 1991; Sriramarao and Bourdon, 1993a). Determined by cell attachment to proteolytic fragments and fusion proteins, TN contains at least four separate cell-binding sites that mediate contrasting adhesive and counteradhesive effects (Friedlander et al., 1988; Prieto et al., 1992). A region containing an arginine-glycine-aspartic acid (RGD) sequence (TNfn3) in the fibronectin type III repeats II-VI mediates cell attachment and spreading and is recognized by the αvβ3 integrin. Another adhesive region in the fibrinogen domain mediates attachment in the absence of cell spreading. The EGF-like repeats and the fibronectin repeats VII-VIII show counteradhesive properties (Aukhil et al., 1993; Prieto et al., 1992).

The intact TN molecule promotes attachment of several cell types, but cell spreading usually is retarded. Attachment to other adhesive proteins of the extracellular matrix (ECM), such as fibronectin, can be inhibited with soluble and insoluble TN probably via a nonspecific, steric covering of binding sites on the other ECM proteins (Lightner and Erickson, 1990). Sriramarao et al. (1993b) showed that attachment of endothelial cells to intact TN could be blocked up to 80% using either anti-α2 or anti-β1 antibodies; all attachment was abrogated by

combining anti- $\beta 1$ and $\alpha v\beta 3$ antibodies (Sriramarao et al., 1993b). The binding site on TN for the $\alpha 2\beta 1$ integrin remains undefined. FG human pancreatic carcinoma cells, which do not express $\alpha v\beta 3$ integrins, utilize two other αv -containing receptors, $\alpha v\beta 5$ and $\alpha v\beta 6$, as cell adhesion receptors to TN (Prieto et al., 1993). It appears that $\alpha v\beta 6$ binds to the same RGD site as $\alpha v\beta 3$. Recently, a newly described integrin receptor, $\alpha 9\beta 1$, which has been identified in a number of cell types outside the CNS, has been shown to bind to the third fibronectin type III repeat of intact chicken TN. However, the binding site seems to be distinct from the RGD site recognized by integrin $\alpha v\beta 3$ (Forsberg et al., 1994; Yokosaki et al., 1994).

Cellular chondroitin sulfate proteoglycans such as neurocan, glypican, phosphocan, and phosphocan KS also show high-affinity binding to a single site on TN in vitro, possibly Tnfbg (Aukhil et al., 1993), and may act as cellular receptors (Grumet et al., 1994; Vaughan et al., 1994). Additionally, receptor type tyrosine phosphatase β (RTP β) binds specifically to TN (Barnea et al., 1994). F11/contactin, a member of the Ig superfamily cell surface adhesion molecules, also binds to TN; this receptor is highly expressed on neurons (Zisch et al., 1992). Recently, annexin II, an intracellular and membrane-associated 35 kDa receptor, has been shown to bind to a spliced isoform of a TN fibronectin type III repeat (TnfnAD); its functional significance as a TN receptor is still unknown (Chung and Erickson, 1994). This complexity of cellular receptors able to recognize distinct binding sites on TN may explain the diversity of biological functions mediated by this ECM molecule including adhesive and counteradhesive cell interactions.

Significant amounts of TN are deposited in the matrix of human tumors, including gliomas in vivo and in vitro (Higuchi et al., 1993; Ventimiglia et al., 1992). Zagzag et al. (1995) recently showed that cellular and interstitial deposition of TN is enhanced in higher grade astrocytomas, and it is detected at the invasive edge of these tumors surrounding infiltrating tumor cells. Tenascin was also strongly associated with hyperplastic blood vessels suggesting a role for it in the neovascularization of these tumors (Zagzag et al., 1995). Glioma cells show high rates of specific migration on surfaces coated with TN in vitro (Giese et al., 1995). The role of this ECM protein in brain invasion by glioma cells remains unknown.

Whereas other investigators have studied the different possible cell binding ligands present on the tenascin molecule by the use of recombinant proteins or selective proteolytic digestion of the native protein, this study focuses on the relative biological consequences of cells responding to these different ligands on intact tenascin. Our results demonstrate that astrocytoma cells, by the constitutive expression of at least two separate integrins that bind ligands on tenascin, integrate the cell's response to the protein based on the density of the different ligands. At low density of tenascin the pro-adhesion and pro-migration responses dominate; when the density of this same multi-ligand protein increases, anti-adhesive and anti-migratory responses are manifest by the cells.

MATERIALS AND METHODS

Antibodies and ECM proteins

Anti- $\beta 1$ integrin antibody (AIIB2) is biologically active in blocking

the ligand-binding site of $\beta 1$ -containing integrins (kindly provided by Caroline Damsky, University of California, San Francisco) (Hall et al., 1990). Anti- αv antibody (Chemicon International, Temecula, CA) recognizes the $\alpha v\beta 3$ and $\alpha v\beta 5$ receptors and inhibits the attachment of platelets to vitronectin (De Vries et al., 1986). Merosin and TN were obtained from Chemicon International; vitronectin was purchased from Collaborative Biomedical Products (Bedford, MA). ECM proteins were reconstituted at 100 $\mu\text{g/ml}$ and stored at 4°C. Dilutions of stocks were made immediately prior to use. Analysis of TN by reducing sodium-dodecyl-sulfate-polyacrylamide electrophoresis in our lab showed a single dominant band at 220-230 kDa (Taylor et al., 1989). ELISA analysis for fibronectin and laminin contaminants in TN was negative (<0.01 $\mu\text{g/ml}$ at TN concentration of 100 $\mu\text{g/ml}$). Substrates of the ECM proteins were prepared by passive coating of tissue culture plastic surfaces using concentrations of the proteins as noted (Aukhil et al., 1993). The culture surfaces were allowed to stay in contact with the proteins at 37°C for 1 hour, followed by three rinses with PBS. Subsequent studies conducted using substrates generated by coating overnight at 4°C (Joshi et al., 1993) indicated that either method led to similar results. ELISA for passively coated surfaces (Slack et al., 1991) indicated that a stoichiometric relationship existed between the concentration of TN used to coat the well and the quantitative binding of monoclonal antibodies to TN ligands (data not shown).

Cell culture and conditioned media

The SF-767 glioma-derived cell line (Berens et al., 1990) was propagated in monolayer culture using minimum essential medium (MEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT). This cell line stains positively for glial fibrillary acidic protein (GFAP). Immunostaining of the extracellular matrix of this cell line for TN failed to demonstrate its presence relative to positive control glioma cell lines (*not shown*). The cell line was free of mycoplasma contamination as assessed by PCR (Stratagene, LaJolla, CA).

All cell migration assays and growth experiments were performed using autologous conditioned medium (CM). To prepare CM, cells were cultured until approximately 70% subconfluent; the medium was replaced by fresh MEM containing 10% FCS and the cultures were allowed to condition their medium for an additional 5 days. Supernatants were harvested, aliquoted and stored at -20°C. The effectiveness of the supernatant to support cell proliferation was tested by performing standard six-day growth curve experiments.

Cell adhesion assay

Cells were tested for adhesion using a method modified from that described by Kramer et al. (1989). Briefly, cells were harvested from monolayer culture and deposited into microtiter wells (50,000/well) (Becton Dickinson & Co., Lincoln Park, NJ). The microtiter plates were incubated for 30 minutes on ice, then at 37°C for 60 minutes to allow adhesion. The plates were then subjected to vigorous agitation (350 rpm for 6 minutes on a horizontal rotator), after which nonattached cells were removed by aspiration and rinsing with phosphate-buffered saline (PBS). Attached cells were fixed in 1% glutaraldehyde and stained using crystal violet (0.1% in H₂O). Absorbance of stained nuclei was quantified using spectrometry at 540 nm (BioTek Plate Reader, Winooski, VT). Replicates from three wells were averaged. The number of attached cells is reported as absorption units. In experiments using anti-integrin antibodies, the antibody was added to the wells before the cells were plated.

Cell migration assay

A recently developed monolayer migration assay (Berens et al., 1994) was used to quantify locomotion of astrocytoma cells on ECM. Eight-chamber LabTek slides (Miles Scientific, Naperville IL) were coated with different concentrations of TN by passive absorption as described above. MEM (200 μl) with 10% FCS was added to the slide

chambers. Sterile custom-produced sedimentation cylinders (G & C Glass, Gilbert, AZ) with an inner lumen diameter of 1 mm and an outer diameter of 7 mm were placed into the center of each chamber. Cells were harvested from monolayer culture and seeded in a volume of 1 µl MEM (2,000 cells) into the central lumen of the sedimentation cylinder. Slides were placed on ice for 60 minutes and then incubated for 12 hours at 37°C in a humidified atmosphere with 5% CO₂. Complete attachment of all cells was confirmed under an inverted microscope and the cylinders were removed. All medium was gently aspirated and 0.4 ml of fresh MEM with 10% FCS and 25% CM was added. The slides were maintained at 37°C except when briefly examined by inverted microscope. The circular area occupied by attached cells in each well was imaged using a camera (Optronics Engineering, Goleta, CA) attached to an inverted microscope (×2.5 objective, Axiovert 35, Zeiss, Germany) and digitalized for quantification with an image analysis system (Vidas 2.1, Kontron Elektronik, Germany). Object sizes were measured as the radius in µm of best fit circles around the area covered by cells. Serial images were captured after the cylinders were removed and for as long as 40 hours thereafter. Quantitative migration scores were calculated as the increase of the radius beyond the initial radius of the object. These measurements represent net changes in the geographical distribution of the cell population and do not reflect movement of individual cells. All migration studies included measurements of cells deposited on a nonspecific protein coating of bovine serum albumin (BSA; Sigma, St Louis, MO). The rate of migration (slope of the increase in cell population radius divided by time) was used as a reference for nonspecific migration. Therefore, specific migration rates (see Figs 4 and 5) are the difference between the migratory response of cells on different ECM proteins relative to that on BSA. Positive values of specific migration indicate motility rates faster than on BSA, while negative rates represent cell movement that is slower than that seen when cells are on BSA. Specific migration rates less than zero suggest active suppression of cell motility.

Biotinylation and immunoprecipitation of integrins

Cells in monolayer culture were rinsed in DPBS at 4°C labeled with Sulfo-NHS-biotin/DMSO (0.5 mg/ml in DPBS) twice for 15 minutes at 25°C (Rosen et al., 1992). The monolayers were rinsed

twice in DPBS and then lysed at 4°C in extraction buffer (1% Triton X-100 in 50 mM Tris, 150 mM NaCl, 10 mM EDTA containing 10 µg/ml pepstatin, leupeptin, and aprotinin). The lysate was centrifuged for 20 minutes at 10,000 rpm and the supernatant precleared with Protein G Sepharose 4B (Sigma). A 250 µl fraction of the supernatant was incubated at 4°C with 10 µl of anti-integrin antibody and 50 µl of Protein G Sepharose 4B for 60 minutes. The immunoprecipitate was washed in extraction buffer three times for 10 minutes. Samples were prepared in reducing and nonreducing SDS buffer (95°C, 5 minutes), separated by 7.5% PAGE, then blotted onto nitrocellulose (PhastSystem, Pharmacia, Piscataway, NJ) for immunostaining (FastBlot, Pierce, Rockford, IL). Biotinylated proteins were identified using avidin-conjugated alkaline phosphatase and chromogenic development with 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (BCIP)/nitro blue tetrazolium chloride (NBT) (Pierce).

RT-PCR of integrin mRNA

Reverse transcriptase (RT) synthesis of cDNA from 5 µg of total RNA was done using a First Strand Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. cDNA aliquots (5 µl) were used for polymerase chain reaction (PCR). Primer pairs for each integrin subunits (Table 1) were derived from literature references or were designed based on published integrin cDNA sequences with the assistance of Oligo 4.0a primer analysis software (National BioScience, Inc., Plymouth, MN). Amplification of PCR products (5 µl of RT product, 2.5 µl of each primer, 0.5 µl of Taq polymerase (Promega, Madison, WI), and 2 µl of nucleotides (Perkin Elmer, Norwalk, CT) in 10 µl of 10× buffer, total volume of 100 µl) was allowed to run for 20 to 40 cycles (1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C) (MJ Research, Inc., Watertown, MA); aliquots (8 µl) were collected every two cycles, run in 2% agarose gels, and stained with ethidium bromide. The absorbance values of the product bands captured on Polaroid 665 film were determined by scanning with visible light (Beckman DU-70) and by calculation of peak area using PeakFit (Jandel Scientific, San Rafael, CA). The quantitative measurements are reported as the interpolated number of cycles needed to generate a product of peak area equal to 10 absorbance unit·mm.

Table 1. Primer sequences for PCR analysis of integrin subunit expression

Gene	Oligonucleotide	Annealing temperature	Size of fragment (bp)	Reference
Integrin β1	5' AAT GGG AAC AAC GAG GTC ATG GTT 3' 5' TTG TGG GAT TTG CAC GGG CAG TAC 3'	60°C	300	Bates et al., 1991
Integrin β3	5' TGC TCA TTG GCC TTG CCG CCC TGC 3' 5' TGA TCT GAG GAT GAC TGC TTA TCA 3'	60°C	200	Bates et al., 1991
Integrin β5	5' CAC TCT GGG AAA CCT GA 3' 5' TTG TTG AAG GTG AAG TCC A 3'	55°C	425	Oligo 4.0a from sequence in Argraves et al., 1987*
Integrin β6	5' TTC TCA AAG GAT GGT TCT G 3' 5' GCA ATC TGT GGA AAG GTC TA 3'	55°C	378	Oligo 4.0a from sequence in Sheppard et al., 1990
Integrin β8	5' CAG GTG ATA CTA CAA TGG 3' 5' CAG CAG CTA TGT GAG TCA 3'	55°C	372	Oligo 4.0a from sequence in Moyle et al., 1991*
Integrin α2	5' TGG GGT GCA AAC AGA CAA GG 3' 5' GTA GGT CTG CTG GTT CAG C 3'	60°C	541	Milam et al., 1991
Integrin α5	5' GCG CTC CAC TGT ACA GCT G 3' 5' CAG CAA GTC ATC CAG CCC G 3'	60°C	564	Milam et al., 1991
Integrin α9	5' GCC TAC AAT GCC CAC TTT 3' 5' CAT GAA GGC TCC AAC AGT 3'	54°C	275	Oligo 4.0a from sequence in Palmer et al., 1993*
Integrin αv	5' GAG CAG CAA GGA CTT TGG G 3' 5' GGG TAC ACT TCA AGA CCA GC 3'	60°C	619	Milam et al., 1991

*Product verified by sequence analysis.

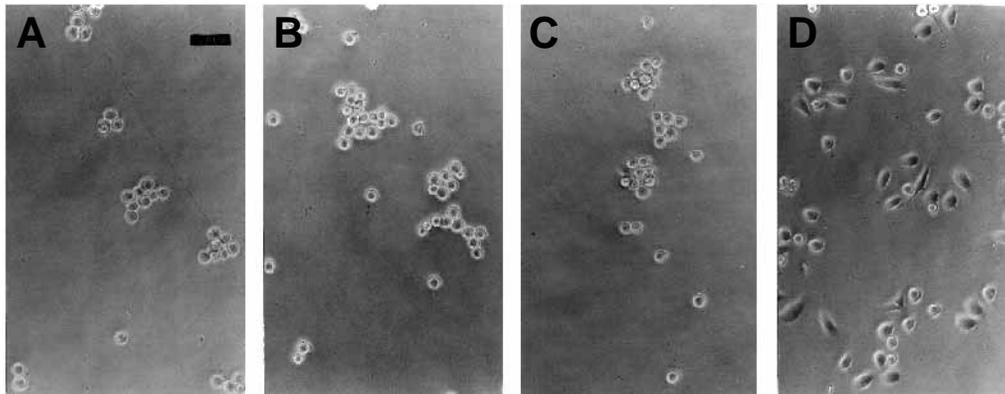


Fig. 1. Cell spreading of human glioma cells, SF767, on TN. Cells were harvested from monolayer culture and seeded on surfaces coated with (A) BSA and TN solutions (B) 0.1 µg/ml, (C) 1.0 µg/ml, and (D) 10 µg/ml. These images show cell attachment and spreading only on TN at 10 µg/ml after 15 minutes. Bar 10 µm.

RESULTS

Cell adhesion to TN

SF-767 glioma cells spread rapidly on surfaces coated with solutions of TN. Attachment and spreading occurred after 15 minutes on surfaces coated with 10 µg/ml TN solution (Fig. 1), while longer intervals were required before spreading was evident with lower and higher coating concentrations. However, after 90 minutes SF-767 cells had fully spread on all concentrations; this included the elaboration of cell processes.

Quantitative adhesion of SF-767 cells to TN at different concentrations was analyzed using a method described by Kramer et al. (1989). After 60 minutes of incubation, unadhered cells were vigorously removed and adhered cells were stained. Analogous to cell spreading, the cells adhered strongest to concentrations of 10 µg/ml TN-coating solution while lower and higher concentrations were less effective (Fig. 2). TN coatings of 100 µg/ml resulted in 50% fewer cells adhering than at 10 µg/ml. This outcome is considered a counteradhesive concentration of the substrate. A similar biphasic adhesion profile was observed with three other glioma cell lines (*data not shown*).

Coincubation of cells in the presence of anti-β1 antibodies (AIIB2) on surfaces coated with 10 µg/ml TN resulted in dose-dependent inhibition of adhesion (Fig. 3A). Dilutions of the antiserum at 1:80 completely blocked adhesion, and 1:640 dilutions were still effective. When tested on a higher (less adhesive) concentration of TN (33 µg/ml), adhesion was enhanced in a dose-dependent fashion by treatment of the cells with anti-αv antibody (Fig. 3B). At the highest anti-αv treatment (1:20), cell adhesion was equal to that seen when using the optimal adhesive TN-coating concentration (10 µg/ml).

Cell migration on TN

We have previously reported that five glioma cell lines showed specific migration on surfaces coated with TN (Giese et al., 1995). For three of those cell lines, TN was the most permissive purified ECM substrate among a panel that included merosin, fibronectin, collagen, and vitronectin. In this study the migration rates of SF-767 glioma cells were analyzed on surfaces coated with TN, merosin or vitronectin at concentrations that ranged from 1 to 100 µg/ml (Fig. 4). The migration rates on each ECM protein were normalized to the motility on BSA; normalized values are referred to as 'specific migration'. The glioma cells responded to increasing concentrations of

merosin with increasing migration rates reaching a maximum specific rate of 510 µm/day at concentrations higher than 33 µg/ml coating solution. On TN, SF-767 cells showed maximum migration rates near 400 µm/day using 3.3 µg/ml coating solution. Contrastingly, migration on a coating concentration of 10 µg/ml TN was much less than on lower concentrations. Concentrations of TN higher than 10 µg/ml inhibited migration relative to the motility on BSA. Vitronectin only marginally supported cell motility.

To investigate the role of specific integrin subunits in migration on TN, anti-β1 or anti-αv antibodies were added to migrating cells during migration, then fresh medium was returned to the cells (Fig. 5). On TN surfaces prepared using 3.3 µg/ml (Fig. 5A and B), anti-β1 antibodies at a dilution of 1:160 almost completely arrested cell migration; migration resumed to pretreatment rates when the antibody was removed (Fig. 5A). Treatment of cells migrating on BSA, with anti-β1 antibodies did not alter the cell motility rate (insert of Fig. 5A), demonstrating that antibody treatment affects the specific integrin-mediated migration. Washing out the antibody restored migration to pretreatment rates. The migration arrest on TN caused by blocking the integrin 1 subunit was dose

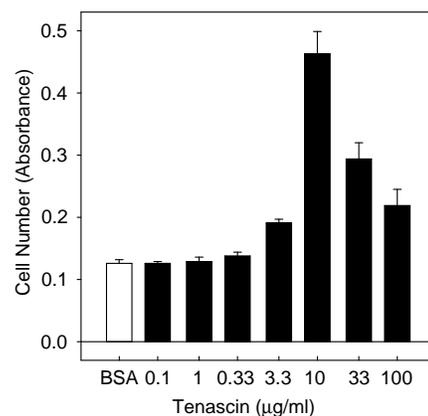
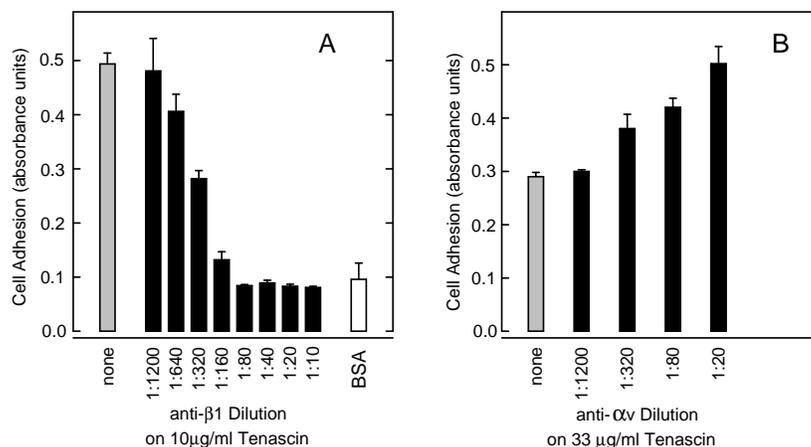


Fig. 2. Effect of increasing coating concentrations of TN on adhesion of human glioma cells. Microtiter plate wells were coated passively with 0.1, 0.33, 1.0, 3.3, 10.0, 33.0 and 100.0 µg/ml TN solutions. Cells were deposited and incubated for 60 minutes to allow specific cell adhesion. Nonadherent cells were removed by shaking and rinsing. Cell number was determined by crystal violet absorbance spectroscopy of stained nuclei. Bars, mean of triplicate determination; error bars, s.d.; BSA, bovine serum albumin.

Fig. 3. Effect of anti-integrin antibodies on glioma cell adhesion to TN. (A) Microtiter wells were coated with the most adhesive concentration of TN (10 $\mu\text{g/ml}$) and anti- $\beta 1$ antibodies were added to the wells before the cells. (B) Microtiter wells were coated with a less adhesive concentration of TN (33 $\mu\text{g/ml}$) and anti- αv antibodies were added to the wells. Bars, mean of triplicate determination; error bars, s.d.



dependent on the blocking antibody (Fig. 5B). Increasing amounts of AIIB2 antibody from 1:640 to 1:160 increasingly arrested cell migration on TN.

Cells migrating on surfaces coated with 10 $\mu\text{g/ml}$ TN increased their migration rate approximately twofold following treatment with anti- αv antibodies at a dilution of 1:10 (Fig. 5C). Migration of SF-767 cells on 10 $\mu\text{g/ml}$ TN substrate could be accelerated in a concentration-dependent manner by increasing titer of the anti- αv antibody (Fig. 5D).

Identification of integrins

The levels of integrin subunit mRNA expressed by SF-767 cells were analyzed using semiquantitative RT-PCR (Fig. 6). The values represent the number of PCR cycles needed to generate a reaction product showing an area of 10 absorbance unit \cdot mm in the densitometric scan of gels. Among the β subunits, $\beta 1$ was predominant, but lower mRNA levels also were detected for $\beta 5$ and $\beta 6$. PCR product for $\beta 3$ and $\beta 8$ was detected only after more than 40 cycles of amplification. All α subunits implicated as dimer components of TN receptors, with the exception of $\alpha 9$, were detected by RT-PCR; $\alpha 2$ and αv were the most abundantly expressed messages.

Immunoprecipitation using anti- $\beta 1$ or anti- αv antibodies showed the presence of a bands at 130 kDa or 165 kDa (respectively) as anticipated, but αv did not co-precipitate with $\beta 1$, nor did $\beta 1$ co-precipitate with αv (data not shown). These data indicate that these two subunits are not dimerized as a single integrin receptor.

DISCUSSION

The adhesive, migratory, proliferative and survival behaviors of cells are highly responsive to ECM proteins (Meredith et al., 1993). Cell-matrix interactions also are responsible for pathological behaviors such as tumor invasion and metastasis (Liotta and Stetler-Stevenson, 1991). Biological ECMs are a complex composition of different proteins and glycosaminoglycans that are recognized by specific cell-membrane receptors (Hay, 1991). The relative levels of the different ECM proteins introduce the prospect that both qualitative and quantitative variables are involved in a cell's response to the matrix.

The diversity of receptors for specific ECM proteins may lead to variable or contrasting responses of different cells to

the same protein. For example, TN is recognized by at least seven different integrin heterodimers. Selective expression of different integrins may account for the ability of neurons to extend processes on TN while glial cell migration is retarded on this substrate (Wehrle-Haller and Chiquet, 1993).

Tumors of glial lineage are described as containing TN in their extracellular matrix, (Higuchi et al., 1993; Ventimiglia et al., 1992) and its distribution is colocalized with invading glioma cells (Zagzag et al., 1995). In vitro, TN supports the migration of different glioma cell lines (Giese et al., 1995). Our present results indicate that TN promotes both cell adhesion and migration, but this response is biphasic over a concentration range of 0.1 to 100 $\mu\text{g/ml}$. Previous studies using laminin, collagen type IV, fibronectin and vitronectin demonstrated that adhesion and migration of glioma cells follow dose-dependent relationships, eventually reaching plateaus (Berens et al., 1994;

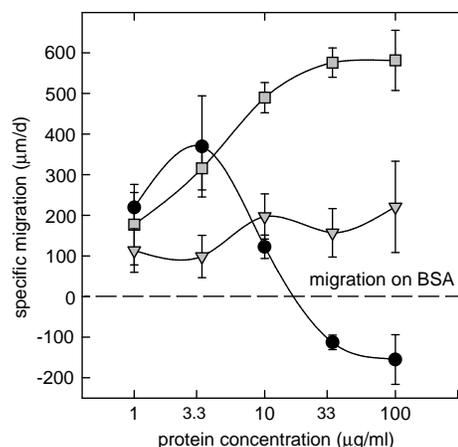
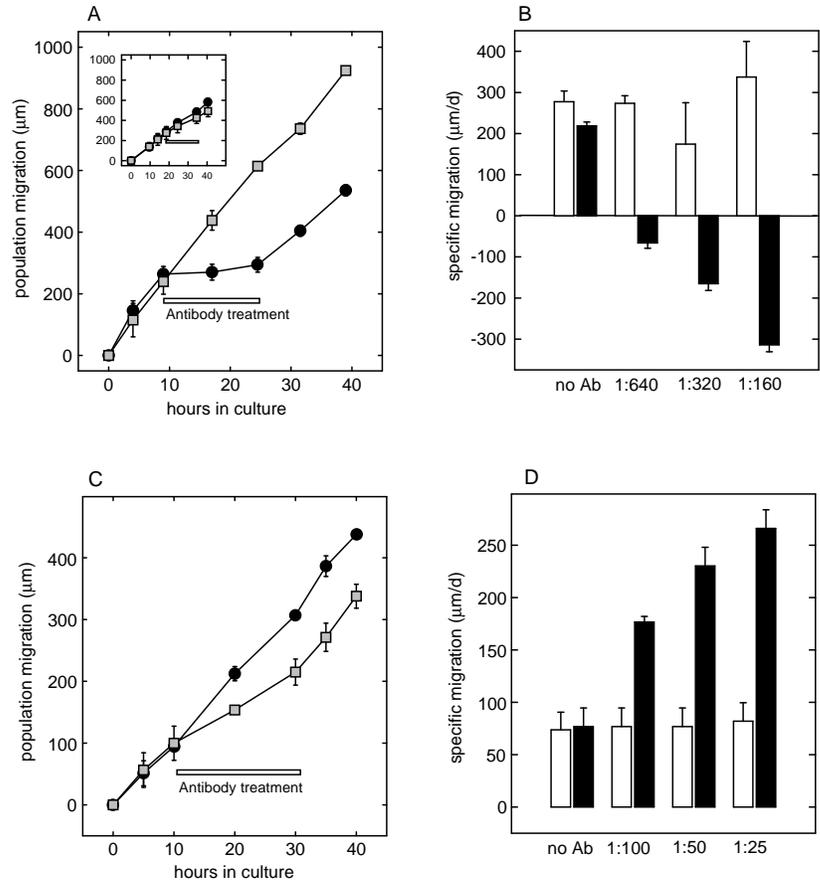


Fig. 4. Effect of increasing coating concentrations of TN, merosin and vitronectin on migration of human glioma cells. Wells of 8 chamber slides were coated with protein solutions as indicated and cells were allowed to migrate for 48 hours. The specific migration response to a protein substrate was calculated as the difference between the migratory rate of the cells on the ECM protein and of cells on BSA. Positive values arise when cells move faster on an ECM protein than on BSA; negative values indicate that the cells moved more slowly on the ECM than on BSA. Migration on increasing concentrations of TN, ●; merosin, □; and vitronectin, ▽. Points, mean of triplicate determination; error bars, s.d.

Fig. 5. Effect of anti-integrin antibodies on migration of human glioma cells on TN. (A) Cells were allowed to migrate on a permissive coating concentration of TN (3.3 $\mu\text{g}/\text{ml}$). Anti- $\beta 1$ antibody at a dilution of 1:160 was added to the cultures after 10 hours of incubation. The cultures were rinsed and antibody-free medium was added after an additional 12 hours. Removal of antibody led to the return of the migration rate to pretreatment level. Anti- $\beta 1$ treated population, \bullet ; mock-treated control, \square . Horizontal bar, presence of Ab. Inset: migration on BSA-coated surfaces. (B) Concentration dependence of migration inhibition by anti- $\beta 1$ antibodies. Migration rates ($\mu\text{m}/\text{day}$) were calculated before (open bars) and after (solid bars) addition of the antibody at the dilutions indicated. Data points, mean of triplicate determination; error bars, s.d. (C) Cells were allowed to migrate on a higher coating concentration (10 $\mu\text{g}/\text{ml}$) of TN that was less permissive for cell movement. Anti- αv antibody at a dilution of 1:25 was added to the cultures after 10 hours of incubation. The cultures were rinsed and antibody-free medium was added after an additional 20 hours. Anti- αv treated population, \bullet ; mock-treated control, \square . Horizontal bar, presence of Ab. Data points, mean of triplicate determination; bars, s.d. (D) Concentration dependent stimulation of cell motility by increasing titer of anti- αv antibody. Open bars show migration rate of cells during mock antibody treatment (fresh medium), solid bars are the rates in the presence of Ab. Values are the mean of five replicates; bars, s.d.



Giese et al., 1994). The biphasic effect of TN may therefore be a unique characteristic of this ECM protein. Both theoretical and empirical arguments for cell migration showing a biphasic response to ECM protein ligand density have been forwarded (DiMilla et al., 1991, 1993; Spring et al., 1989; Wu et al., 1994). These arguments focus on the characteristics of a single receptor/ligand interaction coupled with the dynamics of motile forces. In contrast, we report novel data on the integrated response of astrocytoma cells to one ECM protein perceived by two separate receptors.

The antibody-blocking studies reported here indicate that glioma cell adhesion and migration on TN are mediated by $\beta 1$ -containing integrins. The degree of inhibition by anti-integrin $\beta 1$ antibody treatment was more substantial than reported by others (Sriramarao et al., 1993b). Previously, we have correlated the relative rates of cell motility on different substrates positively with the relative degree of cell adhesion to those substrates (Giese et al., 1994). This finding indicates that motility depends on the binding of specific receptors to their ligands on the substrate (Stein and Bronner, 1989). As an extension of this observation, we report that treatment of glioma cells with antibodies directed at the extracellular region of the αv subunit leads to an increased cell adhesion at otherwise less adhesive (higher) concentrations of TN. Treatment with anti- αv antibodies also enhances the migratory response. This finding adds an additional consideration to the biphasic response of migration due to increasing ligand density proposed by DiMilla et al. (1991, 1993) wherein heightened attachment to the substrate accounts for lost motility. Our

results show that signals from different integrins are integrated by the cells to manifest as a composite effect.

These results indicate that the cell recognizes the substrate via at least two separate integrins and that these two receptors signal contrasting biological responses by the cell. The glioma cells' migratory behavior on TN is a summation of the signals from the two different receptors, one a $\beta 1$ - and the other an

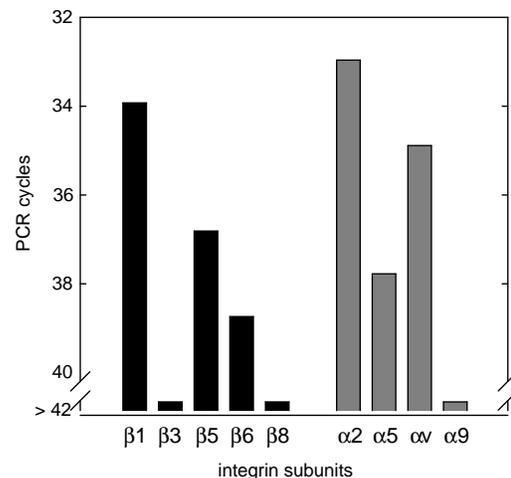


Fig. 6. Semiquantitative profile of integrin subunits expressed by SF-767 cells determined by RT-PCR. Interpolated PCR cycle numbers needed for a primer pair to generate a product of 10 absorbance unit/mm peak area. Profiles are the result of triplicate amplifications.

αv -containing heterodimer. In addition to abrogating all adhesion to TN, blocking the $\beta 1$ -containing integrin leads to a lower migration rate than that associated with BSA. We conclude that blocking the receptor that mediates adhesion to TN establishes a setting for the counteradhesive epitope(s) of TN to dominate the net response (Spring et al., 1989). That the migration rate of cells whose $\beta 1$ receptor has been blocked is below the nonspecific cell motility on BSA (Fig. 5A) suggests that there is an active suppression of cell migration mediated by the αv -containing integrin receptor.

In unmanipulated glioma cells, suppressed adhesion and migration on TN are manifest only at the higher coating concentrations. Integrins are reported to be activated based on ligand density or they may show differing affinities for their ligands (Hynes, 1992); consequently, this separation of adhesive and counteradhesive effects of TN likely reflects the physical or biophysical characteristics of the two different receptors. Furthermore, signal transduction by integrins, mediated by focal adhesion kinase (Luna and Hitt, 1992; Clark and Brugge, 1995) or other signaling pathways such as mitogen activated protein (MAP) kinase (Chen et al., 1994; Crews and Erikson, 1993; Egan and Weinberg, 1993) raises the prospect that antimigratory behavior is a specific response determined by the activation of certain integrins. The binding of the cell to different ECM ligands also influences the degree of localization or dispersion of the integrins as focal versus point contacts, with commensurate consequences on cell spreading and migration (Tawil et al., 1993). When merosin (a migration-permissive ECM protein) is mixed with a nonpermissive protein such as vitronectin, glioma cell motility shows a disproportionate large arrest (unpublished data). Interestingly (in the context of the data reported in this manuscript), vitronectin receptors are comprised of αv -containing integrins. We suggest that different integrins bind with different affinities to their ligands and thereby lead to contrasting cell responses (Williams et al., 1994; Diamond and Springer, 1994).

Of the described TN-binding integrins, our results (from PCR and immunoprecipitation experiments) suggest that glioma cell adhesion is most likely mediated through $\alpha 2\beta 1$ integrins, and that $\alpha v\beta 5$ and/or $\alpha v\beta 6$ mediate the counteradhesive and anti-migratory effects on this ECM protein.

Biphasic phenomena regarding cell motility are well described in chemotactic responses of cells, indicating that a ligand gradient is essential for directed response to compounds. At high concentrations of soluble ligand, the receptors are saturated and the cells fail to discriminate the gradient, rendering them unable to establish a leading edge. We report for the first time that substrates of TN are both permissive and nonpermissive (biphasic) for human glioma cell adhesion and migration, dependent on the coating concentration. This response (in contrast to the nonspecific receptor saturation of chemotaxis) is specific, driven by separate integrins that are activated at different ligand densities. Selective activation of integrins mediating the antimotility response of glioma cells may be a means to influence the in situ behavior of these invasive cells.

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