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Tenascin-C Promotes Microvascular Cell Migration and Phosphorylation of Focal Adhesion Kinase[1]

David Zagzag,[2] Bronya Shiff, George I. Jallo, M. Alba Greco, Cy Blanco, Henry Cohen, Juliette Hukin, Jeffrey C. Allen, and David R. Friedlander

Microvascular and Molecular Neuro-oncology Laboratory [D. Z., B. S., G. I. J., C. B., D. R. F.], the Department of Pathology, Divisions of Neuropathology [D. Z.] and Pediatric Pathology [M. A. G.], the Department of Neurosurgery [D. Z.], and the Kaplan Comprehensive Cancer Center [D. Z., M. A. G.], New York University Medical Center, New York, New York 10016; The Fred Hutchinson Cancer Center, Seattle, Washington 98104 [H. C.]; Division of Pediatric Neurology, Children's and Women's Hospital, Vancouver, British Columbia, V6H 3V4 Canada [J. H.]; and the Institute of Neurology and Neurosurgery, Beth Israel Medical Center, New York, New York 10128 [J. C. A.]

ABSTRACT

Enhanced expression of tenascin-C (TN-C) at the invasive edges of glioblastoma multiforme in close association with vascular sprouts, suggests a role for TN-C in microvascular cell migration. To test this hypothesis, we studied the migration of endothelial cells *in vitro*. In an aggregate migration assay, bovine retinal endothelial cells (BRECs) and human umbilical vein endothelial cells spread and migrated similarly on TN-C or fibronectin (FN). In contrast, U251 MG glioma cells migrated less on TN-C than on FN. Morphological features of U251 MG glioma cells on TN-C included poor cell spreading and short processes. In contrast, on FN, U251 MG glioma cells spread and exhibited long radial processes. Using a transmembrane migration assay, we observed that BREC adhesion was similar on TN-C or FN, whereas U251 MG glioma cells adhered better to FN than to TN-C. In addition, BRECs migrated more across the membrane toward regions coated with TN-C than FN, and conversely, U251 MG glioma cells migrated more toward FN than TN-C. Migration of endothelial and glioma cells toward TN-C or FN occurred in a dose-dependent manner and was strongly dependent on cell adhesion. In this assay, ultrastructural study revealed the migrating phenotype of the endothelial cells through the micropores of the membrane and their spread morphology on TN-C. Moreover, *in situ* hybridization revealed specific expression of TN-C in migrating microvascular cells in a cerebral microvascular ring assay. Finally in a phosphorylation assay, TN-C enhanced focal adhesion kinase phosphorylation of BRECs, but not of U251 MG glioma cells, and FN enhanced focal adhesion kinase phosphorylation of both BRECs and U251 MG cells. The expression of TN-C by migrating endothelial cells and the promotion of endothelial cell adhesion and migration by TN-C suggest a potential role for TN-C in pathological angiogenesis.

INTRODUCTION

TN-C[3] is a large secreted (1, 2) oligomeric ECM glycoprotein that is expressed in a regionally restricted pattern in developing brain, cartilage, and mesenchyme and is re-expressed in tumors, wound healing, and inflammation (1–4). TN-C consists of an NH_2-terminal cysteine-rich region involved in oligomerization, followed by linear segments of epidermal growth factor-like and FN type III repeats and a fibrinogen-like COOH-terminal domain (2). Two structurally and functionally different human TN-C isoforms (~200 and 300 kDa) are generated by alternative splicing of the TN-C transcript, with seven type III repeats being included or omitted in the mRNA (1, 5). Although TN-C knockout mice have no major phenotype (6, 7), the family of tenascin proteins (*i.e.,* TN-C, TN-R, and TN-X) is believed to play a role in several cellular processes, including adhesion, migration, and proliferation (1, 8), that are important in angiogenesis (9).

Expression of TN-C is increased up to 4-fold in brain tumors such as GBM compared with normal tissues (4) and is overexpressed in hyperplastic rather than in nonhyperplastic vessels of astrocytomas. This expression correlates with angiogenesis regardless of tumor grade (4). Vascular and glioma cells express TN-C *in vivo* and *in vitro* (10–15).

TN-C expression also correlates with cell migration in the embryo (1, 16) and with glioma cell migration (17). Moreover, data suggest that TN-C is a permissive substrate for vascular cell migration (5, 13, 15, 18), and anti-TN-C antibodies inhibit endothelial cell sprouting *in vitro* (18). It has also been demonstrated that TN-C induces loss of focal adhesions, is a mitogen for confluent endothelial cells, and enhances endothelial cell migration in culture wound assays (15, 19). Enhanced expression of TN-C at the invasive edges of GBM, *e.g.,* in close association with vascular sprouts, suggests a role for TN-C in microvascular cell migration (4, 14). Integrins form a large family of cell surface heterodimeric transmembrane receptors that transduce ECM signals to the cell, including a cascade of tyrosine phosphorylations (20, 21). In many cell types, FAK is the initial protein that becomes tyrosine phosphorylated and plays a central role in mediating integrin function (22). FAK is overexpressed in GBM, especially at the invasive edge of the tumors (23).

To gain further insight in the implication of TN-C in microvascular cell migration, we tested whether TN-C acts as a substrate that promotes microvascular cell migration. To this effect we used two *in vitro* assays: AMA and the TMA. Moreover, as a first step to test how TN-C might be implicated in the regulation of angiogenesis, we also investigated the expression of TN-C in migrating vascular cells, using the CMA. Finally, we investigated whether FAK phosphorylation could be responsible for the enhanced microvascular migration seen on TN-C. We report here that TN-C is a permissive substrate for microvascular cell migration, that TN-C triggers FAK phosphorylation in endothelial cells, and that migrating human cerebral microvascular cells express TN-C.

MATERIALS AND METHODS

Cell Lines. BRECs were cultured in α-MEM supplemented with 10% FCS, 50 μg/ml Endothelial Cell Growth Supplement (Collaborative Biomedical Products, Bedford, MA), and 30 μg/ml heparin (Elkins-sin, inc., Cherry Hill, NJ). HUVECs were grown in DMEM supplemented with 20% FCS, 50 ng/ml acidic-fibroblast growth factor (a kind gift of Dr. Joseph Schlessinger, Department of Pharmacology, New York University), and 100 μg/ml heparin. U251 MG glioma cells were propagated in DMEM supplemented with 10% FCS.

AMA. To prepare aggregates, BRECs, HUVECs, or U251 MG glioma cells growing as monolayers were brought to suspension with trypsin-EDTA. Ten ml of cell suspension in 25-cm^2 ml tissue culture flasks were shaken in defined

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[2] To whom requests for reprints should be addressed, at Department of Pathology Division of Neuropathology, New York University Medical Center, 550 First Avenue, New York, NY 10016. Phone: (212) 263-6449; Fax: (212) 263-8994; E-mail: dz4@nyu.edu.

[3] The abbreviations used are: TN-C, tenascin-C; ECM, extracellular matrix; FN, fibronectin; GBM, glioblastoma(s) multiforme; FAK, focal adhesion kinase; AMA, aggregate migration assay; TMA, transmembrane migration assay; CMA, cerebral microvascular ring assay; BREC, bovine retinal endothelial cell; HUVEC, human umbilical endothelial cell; NBT, nitroblue tetrazolium salt; BCIP, 5-bromo-4-chloro-3-indolylphosphate, toluidinium salt; PL, polylysine.

medium consisting of α-MEM (for BRECs), DMEM (for HUVECs), or DMEM (for U251 MG glioma cells) supplemented with ITS+ (Collaborative Research) in a rotary environmental incubator overnight at 37°C (24–26). The resulting aggregates were collected by low-speed centrifugation and were separated from single cells by sedimentation through a 3.5% BSA-PBS cushion. The aggregates were plated on 26-well HTC(R) glass slides (Cel-Line Associates, Inc., Newfield, NJ) coated with 100 μg/ml TN-C or FN and blocked with 1% BSA and were allowed to attach for 1 h. Aggregates and migrating cells were analyzed by brightfield and phase contrast microscopy as described previously (26). To determine the migration for individually identified aggregates, the same aggregate was photographed at the same magnification at 1, 3, 5, 7, and 22 h after plating. Migration was quantitated as 0.5 times the difference between the diameter of the region occupied by emigrating cells at the appropriate time and the aggregate diameter at the 1-h time point.

TMA (27). To assess the effect of graded concentrations of TN-C, we used cell culture inserts, polyethylene terephthalate, 8 μm micropore membrane (Falcon; Becton Dickinson, Franklin Lake, NJ). In this assay the underside of the membrane was coated with TN-C or FN at concentrations ranging from 0 to 100 μg/ml. After a 10-min incubation at room temperature, the excess substrate was removed by washing twice with HBSS. BRECs and U251 MG glioma cells were obtained from subconfluent plates by treatment with trypsin-EDTA. The cells were resuspended and seeded on the top of the membrane at a concentration of 4×10^4/200 μl. Five hundred μl of medium were added to the lower chamber. After a 5-h incubation at 37°C in 10% or 5% CO_2, the medium from the upper chamber was removed and nonadherent cells were removed by three gentle washings with 1000 μl of PBS each. The remaining cells were fixed in 3% phosphate-buffered formaldehyde for 15 min and stained with 1% toluidine blue-1% borax for 10 min at 37°C. The cells remaining on the top of the membrane as well those that migrated across the membrane were counted; the number obtained represented the number of adhering cells. The cells on the top of the membrane were then removed using a Q-tip, and the remaining cells were counted. The number of remaining cells represented the number of migrating cells. Counting was performed with a compound microscope at ×200 magnification on an area of 0.25 mm^2.

Electron Microscopy. To observe the process of migration through the membrane coated with TN-C, we performed transmission electron microscopy of the cell culture inserts. The inserts were prepared using a modified method from Technical Bulletin 406 (Becton Dickinson). Three h after the cells were plated, the PBS was removed and 3% glutaraldehyde was added to the insert; fixation was allowed to proceed overnight at 4°C. After fixation, the insert and well were washed twice for 5 min each with sodium cacodylate trihydrate-buffered sucrose. Post fixation was carried out by adding sodium cacodylate trihydrate-buffered osmium (1%) solution to the insert and well, allowing secondary fixation to proceed for 2 h. After secondary osmium fixation, the insert and well were washed twice for 5 min each with sodium cacodylate trihydrate-buffered sucrose at room temperature. The intact insert and well were dehydrated by immersion in a graded series of ethanol solutions. The flexible membrane was then removed from the base of the intact insert by a scalpel and was cut into individual specimens, which were transferred to glass dishes containing propylene oxide (100%) to continue the dehydration and clearing process.

The specimens were infiltrated with Spurr (Ted Pella, Inc., Redding, CA) by transferring them from 100% propylene oxide to a mixture of 50% Spurr, then to 100% Spurr embedding medium and placed in an embedding mold. The specimens were cured in the oven overnight at 65°C. The blocks were cut, forming a pyramid with the side of the membrane of interest at the apex, with the membrane oriented perpendicular to the plane of the apex for sectioning through the membrane. One-μm-thick sections were cut for selection of regions of interest. Ultrathin sections were cut with a diamond knife, mounted on coated copper grids, and stained with uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 transmission electron microscope operated at 60 kV.

CMA. We adapted an existing assay (28) for the study of angiogenesis in the central nervous system. Agarose wells were obtained by punching two concentric circles (10- and 18-mm diameter) in an agarose gel (1.5%). The wells were filled with a collagen solution (1 part 10× MEM, 1 part $NaHCO_3$, and 8 parts 1 mg/ml rat tail collagen, type 1; Collaborative Biomedical). One-mm-long human brain cortical arteries were obtained from freshly excised surgical lobectomy specimens by dissecting away leptomeninges and cortical tissue. The vascular segment was rinsed and transferred to the agarose well. After the collagen gels were thoroughly rinsed, the agarose rings were removed, and medium was then added to each explant (microvessel embedded in collagen), which was then kept at 37°C and 5% CO_2.

***In Situ* Hybridization.** To prepare the probe**,** oligonucleotide primers complementary to a 5′ region of exon 1 of the human TN-C gene (primer 1, 5′-CTA GAA TTC CAG CAG CAC CCA GC-3′; primer 2, 5′-CTC AAG CTT CAC CGA ACA CTG G-3′) were designed based on the published sequence (29). With human genomic DNA as a template for these primers, PCR was used to amplify a 231-bp product. The PCR product was cloned into pBluescript II SK+/− (Stratagene Cloning Systems, La Jolla, CA). Two resulting clones, each containing a 0.23-kb insert, were sequenced according to the Sanger method using Sequenase (US Biochemicals, Cleveland, OH) and were found to exactly match a 231-bp fragment of the published sequence of exon 1 of the human TN-C gene (29). Antisense and sense riboprobes were prepared using the digoxigenin RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). The specificity of the probes was verified by Northern hybridization of human fetal brain total RNA (Stratagene). Using the antisense probe, we detected a band of 6–7 kb, which corresponded to the size of human TN-C mRNA. No signals were generated with the sense probe.

Tissue blocks of the CMA were prepared for *in situ* hybridization as described previously (14). Five-μm-thick sections were cut onto γ-methacryloxypropyl trimethoxysilane-coated slides. Sections were permeated with detergents, sodium bisulfide, and 250 μg/ml proteinase K (Boehringer Mannheim), followed by washes in diethylpyrocarbonate-treated sterile water. The sections were then blocked with denatured/sheared salmon sperm DNA (1 mg/ml) and BSA (50 mg/ml) in 2× SSC. Ten ng of the digoxigenin-labeled probes (sense or antisense) were heated in 20 μl of hybridization buffer at 70°C for 5 min. Hybridization was achieved by adding 17 μl of probe and incubating at 42°C overnight. After posthybridization washes, sections were then treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA) at 2 units/slide for 5 min at 37°C, followed by blocking before detection with antidigoxigenin antibody conjugated to alkaline phosphatase at a 1:500 dilution. The slides were then incubated with substrate containing 45 μl of NBT and 35 μl of BCIP. The sections were counterstained with nuclear fast red and examined. Staining was assessed as strong, weak, or not detectable.

FAK Phosphorylation and Matrix Proteins. Soluble proteins (TN-C, FN, or PL) were coated onto polystyrene dishes (Falcon 1008; Becton Dickinson), incubated at 20°C for 30 min, washed three times with HBSS and blocked with DMEM/ITS+ in a tissue culture incubator for 1 h (30). Cells were harvested through brief incubation in trypsin-EDTA and washed twice through centrifugation with PBS containing 0.5 mg/ml soybean trypsin inhibitor. This procedure dephosphorylates FAK (30). Cells were resuspended in DMEM/ITS+, and 5×10^5 cells/plate were added and incubated in the tissue culture incubator for 25 min. Cells were then extracted.

Sample Extraction. All extractions were performed on ice at 4°C. Cell lines were extracted in a lysis buffer consisting of 1% Triton X-100 in PBS with 1.5 mM $MgCl_2$, 1 mM sodium fluoride, 10 mM sodium PP_i, 0.2 mM sodium orthovanadate, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Extracts were stored at −80°C.

Immunoprecipitation. Procedures were performed at 4°C, and washes were in lysis buffer. Four μg of purified anti-FAK polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY) were bound to protein A beads, washed, and then incubated with 75 μg of cell lysate with agitation provided by a nutator. Beads were washed three times and boiled in 1 volume of 2× SDS sample buffer. The supernatant was collected and processed through Western blots.

Morphological Analysis. Images were captured and digitized using NIH Image. Properties used to describe cell morphology included cell shape, spreading, and phase brightness *versus* phase darkness.

Western Blots. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S (Sigma Chemical Co.). Specific proteins were detected by antibodies, including the anti-FAK polyclonal antibody and monoclonal antiphosphotyrosine (Transduction Laboratories, Lexington, KY). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, Il). Detection was performed through a Pierce superSignal West Femto kit, using horseradish peroxidase-linked secondary antibodies (Amersham, Piscataway, NJ).

Table 1 *AMA and TMA results*

	BRECs		U251 MG glioma cells	
	TN-C	FN	TN-C	FN
AMA[a]				
3 h	35.1 ± 7.4[c]	31.5 ± 10.4	11.3 ± 9.3[c]	46.7 ± 11.3
5 h	57 ± 11.6	53 ± 12.3	41.3 ± 26.6[d]	67.5 ± 13.9
7 h	69.3 ± 19.8	61.3 ± 16.3	46.4 ± 14.6[e]	84.2 ± 10.6
22 h	189 ± 72.4	134.4 ± 26.7	153 ± 25.6[f]	214.5 ± 35.9
TMA[b]				
Adhesion	162.6 ± 1.5	150.3 ± 9.7	177 ± 11.7	203 ± 13.2[g]
Migration	158 ± 8.7[b]	113 ± 11.7	152 ± 6.0	186 ± 14.5[i]

[a] In the AMA, the numbers indicate the distance reached by the cells, in μm.

[b] In the TMA, the numbers indicate the number of cells that adhered or migrated through the membrane. Values indicate means ± SD.

[c–i] Significance: [c] $P = 0.0003$; [d] $P = 0.04$; [e] $P = 0.0002$; [f] $P = 0.03$; [g] $P < 0.05$; [h] $P < 0.01$; [i] $P < 0.05$.

Statistical Analysis. Analyses at each time period were performed using *t* test procedures and the selective rejective Bonferroni procedure of Hochberg (31) to control type 1 errors. *P*s were adjusted using the technique of Wright (32). In the AMA, analyses were conducted to test the hypotheses that the distance migrated was associated with cell type and substrate in a time-dependent function. In the TMA, analyses were conducted to test the hypotheses that the number of adhering or migrating cells was associated with cell type and substrate in a dose-dependent function.

RESULTS

AMA. BRECs and U251 MG glioma cells were tested for their migratory behavior on TN-C and FN. Cells were plated on substrates coated with 100 μg of TN-C or FN. The results are summarized in Table 1. No migration was observed after the first hour, during which aggregate attachment occurred (Fig. 1*A*). During the ensuing time, BRECs migrating on TN-C adopted a spread morphology and had elongated processes (Fig. 1*A*). Although BRECs were also well spread on FN, the cells had a tendency to exhibit less processes than on TN-C (Fig. 1*B*). BRECs migrated and reached similar distances on both substrates (Table 1 and Fig. 2). However, glioma cells remained mostly round with short and slender processes and were much less spread on TN-C than on FN (Fig. 1*C*). In contrast, on FN, U251 MG glioma cells became spread and exhibited very long processes, including radial processes at the outgrowth perimeter (Fig. 1*D*). U251 MG glioma cells reached shorter distances than BRECs (Table 1 and Fig. 2). When BRECs were compared for their ability to migrate on

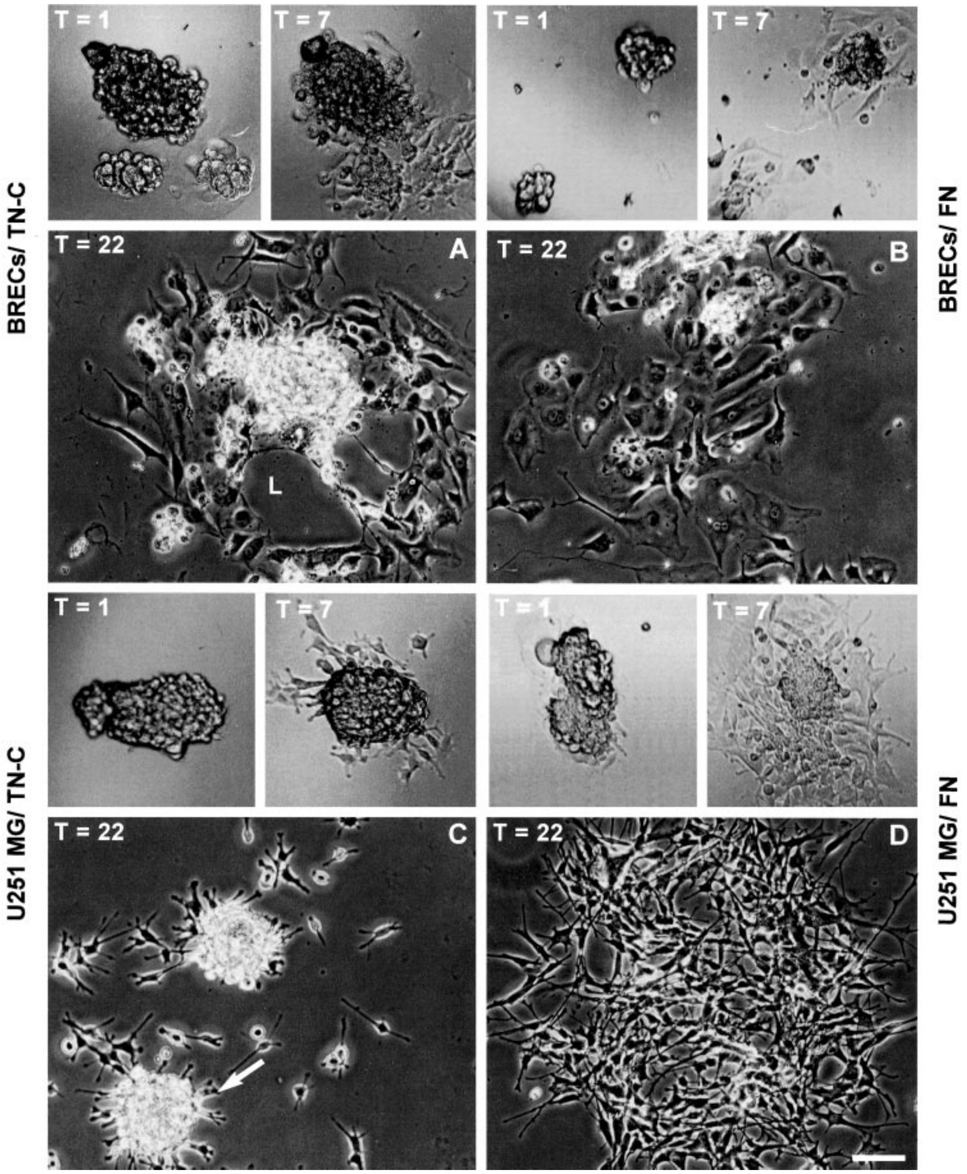

Fig. 1. AMA. Endothelial or glioma cell aggregates were prepared overnight and plated on TN-C or FN (100 μg/ml) as described in "Materials and Methods." Migration was monitored by obtaining micrographs at 1, 7, and 22 h. *Scale bar*, 100 μm. Pairs of micrographs obtained at 1 and 7 h are displayed directly *above* the corresponding 22 h micrograph. Micrographs obtained at 1 and 7 h were taken under oblique illumination, and those obtained at 22 h (*A–D*) were phase micrographs. At 1 h, aggregates exhibited no cellular emigration. At 7 h, early scant emigration could be detected. *A*, migration of BRECs on TN-C. Note the spread morphology adopted by the endothelial cells seeded on TN-C as well as the frequent intercellular contacts that appear to delineate lumen-like structures (*L*). *B*, migration of BRECs on FN. Note the spreading of endothelial cells on FN, as well as the tendency to exhibit ellipsoidal shape on this substrate. *C*, migration of U251 MG glioma cells on TN-C. Note compact aggregates exhibiting few emigrating and poorly spread cells with short processes. The glioma cells tended to remain as aggregates. The *white arrow* in *C* (*T = 22*) indicates the aggregate shown on the *T = 1* and *T = 7* micrographs. There was a "shrinkage" and rounding up of the aggregate (*white arrow*). *D*, migration of U251 MG glioma cells on FN. In sharp contrast, glioma cells scattered readily, exhibiting spreading and long processes.

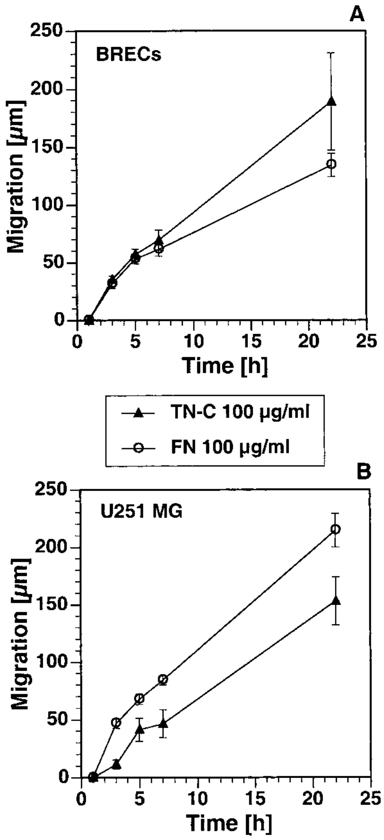

Fig. 2. AMA. AMAs were performed as indicated in "Materials and Methods." Migration of BRECs (*A*) or U251 MG glioma cells (*B*) on TN-C (▲) or FN (○) substrates was determined at 1, 3, 5, 7, and 22 h. No migration was observed during the first hour. Differences in distance migrated on TN-C *versus* FN over a time period from 1 to 22 h were evaluated. For endothelial cells (*A*), no apparent differences in distance migrated were found. However, substrate was a significant predictor of distance traveled by glioma cells over time (*B*); *P*s are included in Table 1. At each of the four time points, distances achieved by glioma cells on TN-C were significantly smaller than on FN. Each data point represents the average of 3–10 observations. Values are expressed as means; *bars*, SE.

TN-C *versus* FN in time course experiments, no significant differences were observed. When such experiments were performed with U251 MG glioma cells, longer distances were observed on FN during the course of the observations (3 h, $P = 0.03$; 5 h, $P = 0.04$; 7 h, $P = 0.0002$; 22 h, $P = 0.03$). Aggregates of endothelial and glioma cells adhered to BSA, but did not spread, and minimal migration was observed (data not shown).

To investigate whether the observed effects of TN-C on BRECs were typical for endothelial cells, HUVECs were tested for their ability to migrate on TN-C or FN. On TN-C substrates (Fig. 3*A*), HUVECS spread and migrated well at levels comparable to those promoted by FN (Fig. 3*B*). These observations support the notion that TN-C promotes spreading and migration of animal and human endothelial cells.

TMA. To assess the effect of gradients of TN-C or FN on migration of endothelial cells, the TMA was used. Graded concentrations of TN-C and FN were prepared to coat the underside of the membranes, and cell migration across the membrane was evaluated. In addition, to assess the contribution of cell-to-substrate adhesion to cell migration, adhesion and transmembrane migration were determined independently. The results are summarized in Table 1. For all cases, cell adhesion and migration curves were remarkably similar, close to superimposable, indicating a strong contribution of cell adhesion to migration in this assay (Fig. 4). For BRECs and U251 MG glioma cells on TN-C and for BRECs on FN, the number of migrating cells was proportional to the coating concentration. For U251 MG glioma cells, FN was roughly 10-fold more potent than TN-C in stimulating adhesion and migration, and the dose-response curve exhibited saturation at ~ 30 µg/ml. As expected, the number of adherent cells varied over a wide range in these dose-response experiments. In contrast to the wide range of adhesion and migration values, the percentage of migrating cells compared with adhering cells was restricted to a high range (63–97%) among the four pairings of cell types and substrates studied. When BRECs were compared for their ability to adhere to TN-C *versus* FN, similar adhesion was observed. When U251 MG glioma cells were tested, FN was a better promoter of adhesion than TN-C ($P < 0.05$). Evaluation of the migration of BRECs by the TMA indicated that TN-C was a better promoter of migration than FN ($P < 0.01$). Finally, in this assay, glioma cells migrated at higher levels on FN than on TN-C ($P < 0.05$).

Electron Microscopy. Ultrastructural analysis of the BRECs in the TMA demonstrated cells attached to the top surface of the membrane and others migrating totally or partially through the micropores to reach the underside coated with TN-C (Fig. 5). In agreement with the phase micrographs in the AMA (Fig. 1*A*), the electron micrographs in the TMA showed spread cells on the TN-C-coated side of the membrane but not on the uncoated top surface of the insert, where the cells preserved their round shape. Migrating cells extended cytoplasmic processes through the micropores of the membrane.

CMA. To further test the involvement of TN-C in vascular cell migration, we used *in situ* hybridization experiments to determine whether migrating vascular cells express TN-C mRNA (14). We used the CMA and observed specific staining in migrating vascular cells (Fig. 6). After a 3-h exposure to the NBT-BCIP substrate, *in situ* hybridization demonstrated strong nuclear and cytoplasmic staining in migrating cells, indicating the presence of TN-C mRNA (Fig. 6*A*). Strong staining was also noted in the endothelial cells lining the vascular lumen. Weaker staining was seen in smooth muscle cells of the media of the same blood vessels. No staining was observed when the sense probe was used (Fig. 6*B*). Moreover, no cellular staining was detected when *in situ* hybridization was performed immediately after transfer of the segment of vessel into collagen and fixation in paraformaldehyde before migration occurred (data not shown).

FAK Phosphorylation. Using the AMA, we observed that BRECs spread well on both TN-C and FN, in contrast to U251 MG glioma cells, which spread only on FN. To interpret these observations, we hypothesized that BRECs would exhibit similar levels of FAK phos-

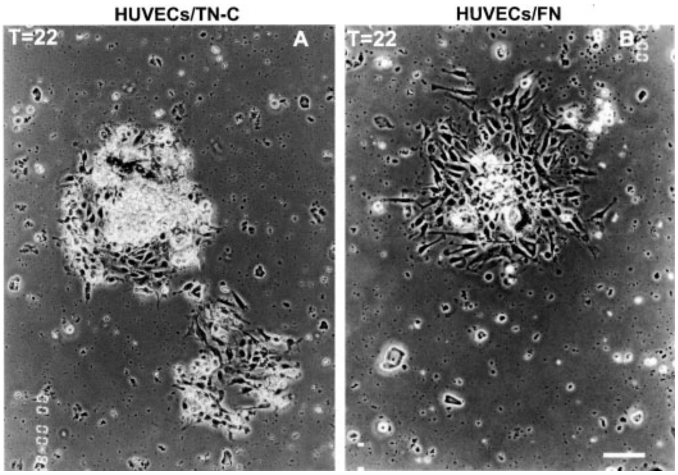

Fig. 3. AMA. HUVECs were aggregated overnight, plated on TN-C (*A*) or FN (*B*), and monitored through photomicrography at 1, 7, or 22 h. The micrographs illustrate the 22-h time point. Note the spread HUVEC morphology and migration on either substrate. *Scale bar*, 100 µm.

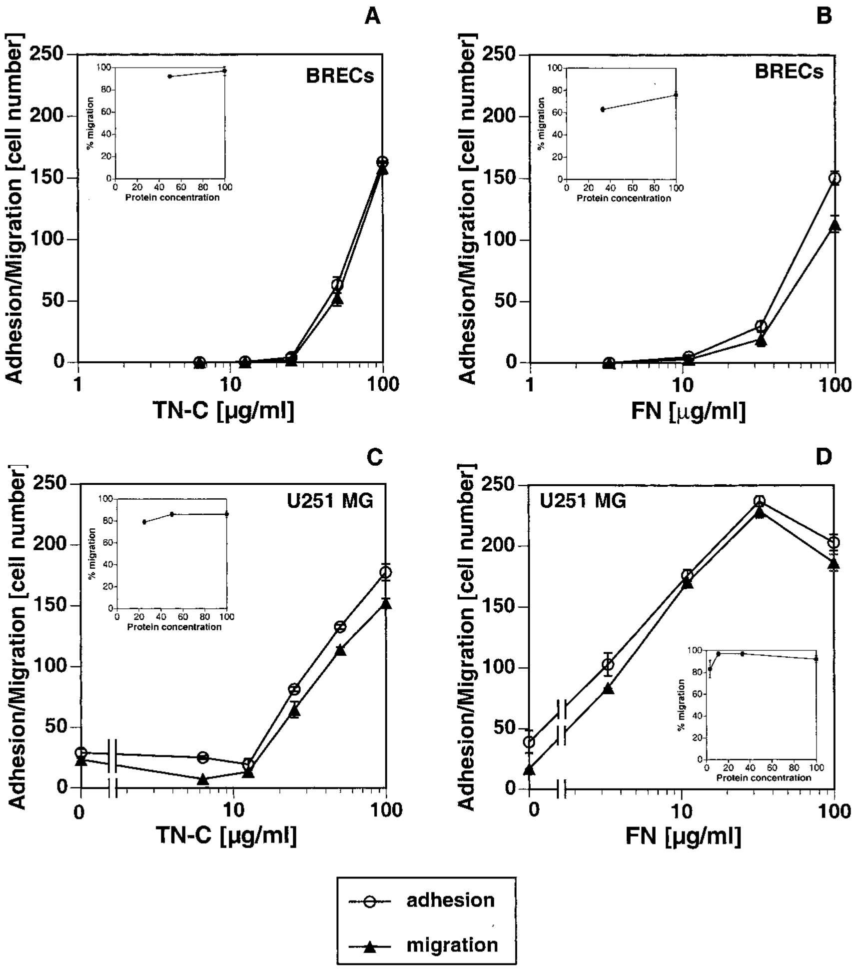

Fig. 4. TMA. Adhesion and transmembrane migration of BRECs and U 251 MG glioma cells on TN-C and FN; dose response. Cell adhesion and migration assays were performed as described in "Materials and Methods." The membrane was coated with 0.0–100 µg/ml TN-C or FN. Both cell adhesion and migration are expressed in cell number units. Adhesion and migration were proportional to protein concentration (*A*, *B*, and *C*) except for U251 MG glioma cells, which exhibited saturation at ~30 µg/ml on the FN substrate (*D*). Note the similarity of the curves in all *panels* for cell adhesion and migration assays. U251 MG glioma cells adhered in the absence of TN-C or FN coating (*C* and *D*), whereas BRECs did not (*A* and *B*). Values are expressed as means; *bars*, SE. Data points represent the average of 4–10 observations. *Insets*, *% migration* indicates the percentage of cells that migrated across the membrane at the indicated protein concentration. The number of migrating cells was normalized to the number of adherent cells and expressed as a percentage. Note that the percentage of migrating cells is essentially constant over the range of protein concentrations tested.

phorylation on both FN and TN-C and that U251 MG glioma cells would exhibit significantly lower levels of FAK phosphorylation on TN-C than on FN. To test this hypothesis, BRECs or U251 MG glioma cells were plated onto TN-C or FN or on PL as a control. After 25 min in culture, images of cells were captured. Cells exhibited characteristic patterns that depended on both cell type and substrate (Fig. 7*A*). U251 cells plated on PL were partially spread, tended to exhibit a triangular shape, and were frequently phase-bright. On FN, they were spread and tended to be round and phase-dark. Consistent with their behavior in the AMA, U251 MG glioma cells plated on TN-C were poorly spread, had short processes, and were mostly phase-bright. BRECs plated on PL did not spread and were phase-bright. On FN, they were frequently well spread, phase-dark, round, and with occasional long processes. Finally, on TN-C, BRECs were spread, elongated, and phase-dark. Immediately after image capture, cells were extracted and analyzed for tyrosine-phosphorylated FAK expression.

Depending on the substrate used, U251 MG glioma cells and BRECs showed various degrees of FAK phosphorylation (Fig. 7). When plated on substrates coated with PL, U251 MG glioma cells exhibited low levels of FAK phosphorylation. In contrast, when U251 MG glioma cells were plated on FN, high levels of FAK phosphorylation were observed. TN-C substrates stimulated low levels of phosphorylated FAK, comparable to those elicited by PL. When BRECs were plated on PL, minimal FAK phosphorylation was observed. In contrast, when BRECs were plated on TN-C or FN, high levels of FAK phosphorylation were observed. No changes were observed in the FAK immunoblots, confirming that the enhanced signals were attributable to tyrosine phosphorylation of FAK.

DISCUSSION

This study provides evidence that (*a*) although endothelial cells adhere, spread, and migrate well, U251 MG glioma cells adhere but

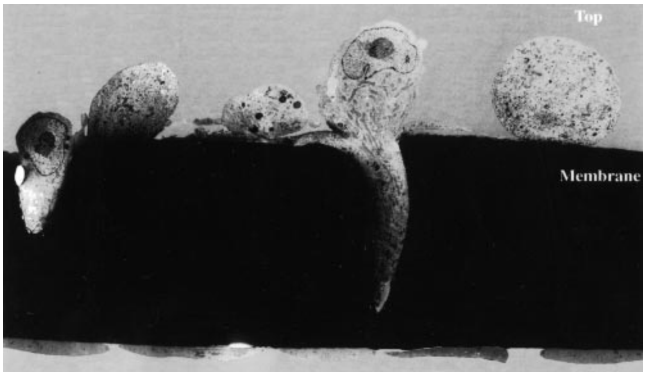

Fig. 5. Transmission electron microscopy. Micrograph showing a monolayer of endothelial cells cultured in a Falcon cell culture insert containing a tissue culture-coated membrane (see "Materials and Methods"). Endothelial cells are seen migrating from the upper surface of the membrane (*top*) through the micropores toward the TN-C-coated underside. Note the round shape of the cells on the top of the membrane in contrast to the spread shape of the cells on the bottom (coated side). Uranyl acetate and lead citrate (original magnification, ×4000).

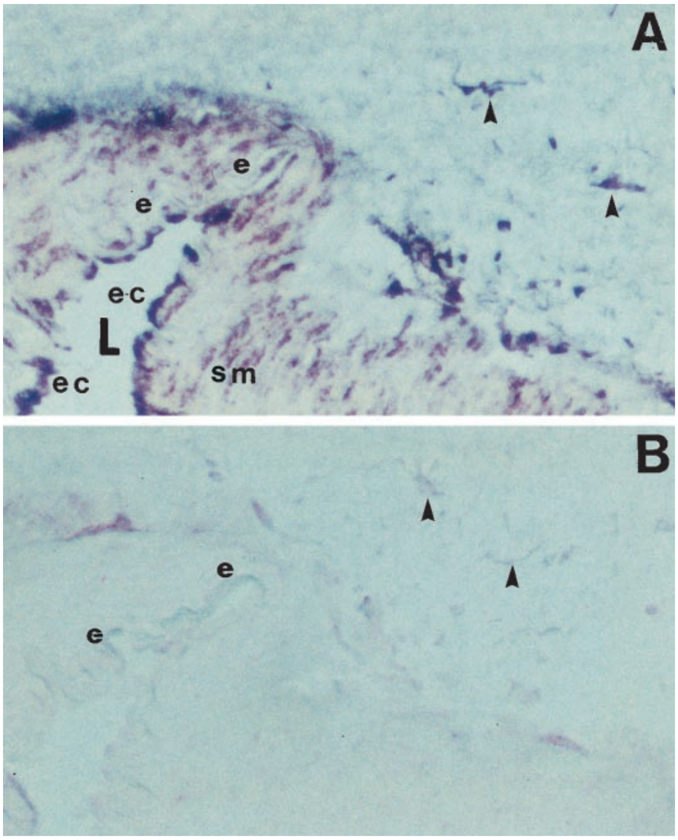

Fig. 6. Expression of TN-C mRNA in the CMA. *In situ* hybridization was performed with a digoxigenin-labeled riboprobe transcribed from a 231-bp PCR product corresponding to a segment of exon 1 of human TN-C. It was detected with antidigoxigenin antibodies conjugated to an alkaline phosphatase enzyme after exposure to NBT-BCIP substrate. The elastic lamina (*e*) is seen as a wavy structure. *A*, after a 3-h exposure to the NBT-BCIP substrate, strong staining of migrating cells (*arrowheads*) is seen with the antisense riboprobe. Staining is also seen in endothelial cells (*ec*) lining the vascular lumen (*L*) as well as weaker staining in smooth muscle cells of the media (*sm*; magnification, ×40). *B*, no staining was detected with the sense probe on a section adjacent to that shown in *A*; intima, media, and migrating cells (*arrowheads*) remained unstained (magnification, ×40).

spread and migrate poorly on TN-C; (*b*) migration of endothelial and glioma cells is remarkably dependent on cell adhesion in the TMA, in which only the bottom of the membrane was coated with TN-C or FN; (*c*) endothelial cells adopt a round cell shape in the absence of matrix protein and acquire a spread morphology on TN-C as seen by ultrastructural analysis; (*d*) *in situ* hybridization demonstrates that migrating vascular cells express TN-C mRNA in a human organotypic model; and finally (*e*) TN-C promotes FAK phosphorylation of BRECs, but not of U251 MG glioma cells, and FN enhances FAK phosphorylation of both BRECs and U251 MG glioma cells.

TN-C and Adhesion. The different effects of TN-C on endothelial or glioma cell adhesion and spreading indicate that cell type is important. For example, endothelial cells adhere better to TN-C than do fibroblasts (33); they also elongate and extend interconnecting processes when plated on TN-C. These features are lacking when endothelial cells are grown on other matrix molecules, including collagen, laminin, and vitronectin (34), suggesting a specific interaction between TN-C and endothelial cells. TN-C enhanced adhesion and spreading of endothelial cells in the assays we used. The observation that TN-C promoted the spread and migration of both BRECs and HUVECs suggests that these behaviors are typical for animal and human endothelial cells. However, TN-C contains adhesive and counter-adhesive binding sites for a variety of cells (35). For example, the third FN-III type repeat of TN-C interacts with $\alpha_V\beta_3$ integrin and promotes adhesion of endothelial cells (33, 34). In contrast, the fibrinogen globe of TN-C binds $\alpha_2\beta_1$ integrin and reduces endothelial cell adhesion (15, 33, 34). Similarly, the counter-adhesive alternatively spliced A-D FN-III domain (which lies between the fifth and sixth FN-III repeats) of TN-C binds with high affinity to the calcium-dependent phospholipid-binding 35-kDa nonintegrin receptor annexin II (36). This results in the disruption of focal adhesions in well-spread endothelial cells and induces cellular rounding, indicating a complex role for TN-C in these processes.

We observed that U251 MG glioma cells adhered but did not spread on TN-C as described previously for neural crest cells or chick embryo fibroblasts (37, 38). Moreover, glioma cell lines migrate poorly on TN-C (26). These include U-87 MG, U-118 MG, U-138 MG, U-373, A-172, and HS 683 (26). Moreover, poor migration was also observed with glioblastoma cells from patients, indicating the biological relevance of our observation using cell lines (26). These results extend the findings obtained to the broader range of glioma cell types exemplified by U251 MG glioma cells. This supports the idea that cell-substrate adhesion and cell spreading are separable processes (39, 40) mediated by distinct ECM domains (41). The difference in the phenotype of the glioma cells on TN-C *versus* FN suggests that cell surface receptors may be critical in modulating cell adhesion and spreading (42, 43). TN-C interacts with other ECM molecules in modulating cell adhesion and spreading. For example, when cultivated on type I collagen gels supplemented with TN-C, human and sheep heart valve interstitial cells adopt a spread morphology and show increased expression of matrix metalloproteinase-2 (44). Similarly, vascular smooth muscle cells maintained on type I collagen and TN-C also spread extensively and form large focal adhesions (45).

TN-C and Migration. TN-C promotes migration of various cell types, including endothelial cells (15), lymphocytes (46), ovarian (47) and laryngeal (48) carcinoma cells, and neural crest cells (37, 49), which are inhibited by anti-TN-C antibodies (18, 19, 36, 50). BRECs and HUVECs migrated and reached similar distances on both TN-C and FN, indicating that TN-C promotes endothelial cell migration, in contrast to inhibitory thrombospondin-rich matrices (18, 19). TN-C is also present at the site of migration of developing embryonic vasculature (2, 51, 52); during corneal development, cells derived from the neural crest and destined to become endothelia migrate exactly along the line of the TN-C-rich stroma (5). Nonsprouting aortic endothelial cells treated with basic fibroblast growth factor and exogenous TN-C, but not with FN, adopt an elongated phenotype and form vascular sprouts (15).

The TMA showed that between 97% and 63% of adhering cells migrated across the membrane among the four pairings of cell types and substrates used. Thus, cell adhesion dominated the migration curve, whereas changes in protein concentration were weakly influential. Despite similarities in adhesion between TN-C and FN for BRECs, the higher level of migration toward TN-C was statistically significant, indicating that factors other than adhesion play a role in migration of endothelial cells.

Using the CMA, we observed specific TN-C staining of migrating human cerebral microvascular cells, suggesting that TN-C expression by vascular cells correlates with their activation and is absent in a "quiescent state" (13). Similarly, TN-C is specifically expressed by sprouting and cord-forming endothelial cells, but not by nonsprouting resting aortic endothelial cells, whereas FN is expressed by both types of cells (15). Mechanisms regulating TN-C expression in vascular cells have only been partially elucidated, *e.g.,* it is sometimes induced by mechanical stretch of vascular smooth muscle cells (53, 54). Several angiogenic factors that promote cellular migration can up-regulate TN-C expression (55). These include acidic (55, 56) and basic (12, 57–59) fibroblast growth factor, platelet-derived growth

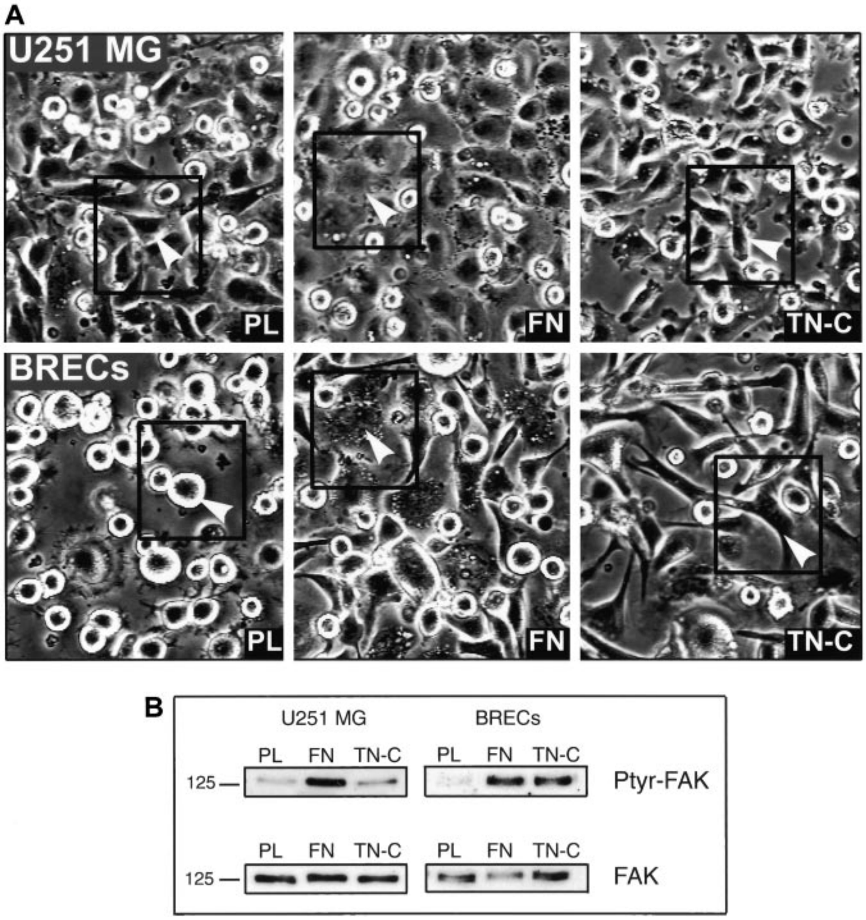

Fig. 7. Phosphorylation of FAK. *A*, morphology. U251 MG glioma cells or BRECs were suspended and allowed to attach to FN or TN-C substrates, as indicated in "Materials and Methods." Images were obtained 25 min after plating. *Top panels* show U251 MG glioma cells. On PL substrates (*left*), glioma cells were typically triangular and partially spread (*arrowhead*). In contrast, on FN (*middle*) the cells were round and well spread (*arrowhead*) and mostly unspread on TN-C (*right*; *arrowhead*). *Bottom panels* depict BRECs. On PL (*left*), BRECs were unspread (*arrowhead*), whereas they were frequently spread on FN (*middle*; *arrowhead*) and both elongated and spread on TN-C (*right*; *arrowhead*). *B*, FAK phosphorylation. Immediately after image capture, cells were extracted and probed for phosphorylated tyrosine on FAK (*Ptyr-FAK*) or FAK as indicated in "Materials and Methods." Note the high levels of Ptyr-FAK in U251 MG glioma cells plated on FN and in BRECs plated on FN or TN-C. *Bar* indicates the migration of FAK in kDa.

factor (60–62), and tumor necrosis factor (60, 63). However, transforming growth factor β, a potent angiogenic factor *in vivo* (64) that up-regulates TN-C (13, 60, 61, 65), inhibits the migration of vascular smooth muscle cells *in vitro* (66). Growth factors may modulate vascular cell migration, at least in part, by controlling the expression of ECM molecules (67) such as TN-C.

TN-C, Integrins, and FAK. In both the AMA and the TMA, TN-C was less potent than FN in promoting migration of U251 MG glioma cells. On a three-dimensional fibrin matrix containing FN, TN-C suppressed actin stress fibers and induced actin-rich filopodia. This distinct morphology was associated with complete suppression of the activation of RhoA, a small GTPase that induces actin stress fiber formation (68). Interestingly, the enhancing effect of TN-C on the migration of glioma cells plated on FN (17) is completely blocked by antibodies to β1 integrin, which interacts with the fibrinogen globe of the TN-C molecule (17). Integrin function depends on interactions with a complex of cytoskeletal proteins that recruit complexes of signaling proteins, including FAK, a nonreceptor protein kinase (69) that often is the initial protein that becomes tyrosine phosphorylated (70). Both TN-C and FN promoted FAK phosphorylation of BRECs, but only FN-enhanced FAK phosphorylation of U251 MG glioma cells provided a mechanistic interpretation for the high levels of migration by BRECs on TN-C and FN. FAK mediates integrin function (22) and associates with the cytoplasmic tail of β_1 and β_3 integrins that can trigger FAK phosphorylation (20, 69). Cells from FAK knockout mice demonstrate decreased migration *in vitro* compared with wild-type cells (71). In addition, evidence linking FAK to angiogenesis include (*a*) interaction with β_3 integrin (20, 69), (*b*) up-regulation by vascular endothelial growth factor (72), and (*c*) the severe defects of both initial vasculogenesis and subsequent angiogenesis found in FAK knockout mice (71), with early embryonic death at E8.0–8.5 (73)

Studies on various nonbrain tumors showed a direct correlation between FAK expression and invasiveness (74). Similarly, we observed FAK overexpression in GBM, especially at the invasive edge of high-grade gliomas (23). Furthermore, hyperplastic vessels demonstrated strong immunoreactivity for FAK. This is important because FAK seems to be involved in cell proliferation, angiogenesis, and apoptosis/necrosis, the hallmarks of GBM, and plays a crucial role in cell migration, a fundamental process involved in the diffuse infiltration of brain parenchyma that makes GBM extremely difficult to cure.

TN-C and Angiogenesis. TN-C promotes endothelial cell adhesion, spreading, and migration (5, 18, 19, 33, 34, 49, 75, 76), which are critical for angiogenesis (9). Additional evidence linking TN-C and angiogenesis includes the following: (*a*) TN-C expression is spatially and temporally up-regulated in many conditions associated with angiogenesis, *e.g.*, in newly formed vessels of granulation tissue in wound healing, but it is not detectable or is markedly reduced in the healed scar (3, 75, 77). TN-C is also expressed during angiogenesis associated with arthritis (78, 79) and neoplastic diseases (80). For example, in human gliomas, TN-C accumulation and TN-C mRNA detection correlate with the degree of tumor neovascularization (4, 14, 81). (*b*) TN-C binds to heparin (82, 83), an important modulator of angiogenesis (84). (*c*) Endothelial cells adhere to TN-C, in part through $\alpha_2\beta_1$ and $\alpha_v\beta_3$ (33, 34), both of which are implicated in angiogenesis (51, 85). $\alpha_v\beta_3$ is critical for basic fibroblast growth factor-induced angiogenesis (26) and is up-regulated in angiogenic vessels of GBM (86); (*e*) TN-X, a member of the tenascin family, interacts with vascular endothelial growth factor, a potent angiogenic factor, to enhance endothelial cell proliferation (87).

In summary, our results suggest a potential role for TN-C in pathological angiogenesis as observed for other ECM molecules, including laminin, FN, collagen, thrombospondin, and SPARC (25, 28, 52, 88, 89). However, because of the early expression of TN-C, it may play an important role in initiating angiogenesis. In view of its particular implication in brain tumors (4, 14) and its critical role in microvascular migration, TN-C may be one of the important ECM molecules in brain tumor angiogenesis. By expressing TN-C, endothelial cells modify the ECM composition, which may facilitate vascular sprouting and migration critical for angiogenesis. Moreover, because TN-C is up-regulated in tumor vasculature of cerebral neoplasms, antibody-directed therapies targeting TN-C and causing tumor infarction (90) may prove to be useful in treating brain tumors.

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