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Integrins mediate adhesion of medulloblastoma cells to tenascin and activate pathways associated with survival and proliferation

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Medulloblastoma spreads by leptomeningeal dissemination rather than by infiltration that characterizes other CNS tumors, eg, gliomas. This study represents an initial attempt to identify both the molecules that mediate medulloblastoma adhesion to leptomeninges and the pathways that are key to survival and proliferation of tumor following adhesion. As a first step in molecule identification, we produced adhesion of D283 medulloblastoma cells to the extracellular matrix (ECM) of H4 glioma cells *in vitro*. Within this context, D283 cells preferentially expressed the $\alpha 9$ and $\beta 1$ integrin subunits; antibody and disintegrin blockade of $\alpha 9$ and $\beta 1$ binding eliminated the adhesion. The H4 ECM was enriched in tenascin, a binding partner for the $\alpha 9\beta 1$ integrin heterodimer. Purified tenascin-C supported D283 cell adhesion. The adhesion was blocked by antibodies to $\alpha 9$ and $\beta 1$ integrin. *In vivo* data were similar; immunohistochemistry of primary human medulloblastomas with leptomeningeal extension demonstrated increased expression of $\alpha 9$ and $\beta 1$ integrins as well as tenascin at the interface of brain and leptomeningeal tumor. These data suggest that tumor-cell expressions of $\alpha 9$ and $\beta 1$ integrins in combination with extracellular tenascin are necessary for medulloblastoma adhesion to the leptomeninges. As a first step in the identification of pathways that mediate survival and proliferation of tumor following adhesion, we demonstrated that adhesion to H4 ECM was associated with survival and proliferation of D283 cells as well as activation of the MAPK pathway in a growth factor deficient environment. Antibody blockade of $\alpha 9$ and $\beta 1$ integrin binding that eliminated adhesion also eliminated the *in vitro* survival benefit. These data suggest that adhesion of medulloblastoma to the meninges is necessary for the survival and proliferation of these tumor cells at the secondary site.

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Leptomeningeal dissemination, the spread of cerebellar medulloblastoma to the surface of the central nervous system (CNS), predicts poor patient outcome.[1,2] The process of leptomeningeal dissemination is distinct from the tissue infiltration that characterizes the spread of other CNS tumors, eg, gliomas.[3] In leptomeningeal dissemination, tumor cells migrate from the primary site to the surface of the brain and spinal cord, adhere to these secondary sites, and establish new colonies.[4] Risk factors for leptomeningeal dissemination have been identified[5–7] and some recent studies suggest that expression of ERBB2, PDGFR-α, and PDGFR-β stimulates the migration of medulloblastoma cells.[6,8] However, the mechanisms of adhesion and survival following spread are not understood.

Adhesion of medulloblastoma cells to the leptomeninges has received little attention since the 1990s. Early investigations suggested a promising *in vitro* model for leptomeningeal dissemination: in a series of articles from 1986 to 1988, Rutka *et al*[9–14] produced medulloblastoma adhesion to the leptomeningeal extracellular matrix (ECM) in a cell culture model. These investigators identified key proteins in ECM, including fibronectin, procollagen III, laminin, and collagen IV; however, the relatively small number of adhesion factors recognized at that time precluded identification of the specific molecules that mediate attachment. In 1991, Wikstrand, Friedman, and Bigner explored adhesion of six different medulloblastoma cell lines to three proteins

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(fibronectin, laminin, collagen IV) and Matrigel, but could not replicate Rutka *et al*'s success in producing adhesion to these proteins.[15] We found no published studies of medulloblastoma adhesion to the leptomeninges after 1991.

Understanding of cell adhesion in the nervous system has progressed significantly during the past 15 years.[16,17] Of particular relevance to leptomeningeal dissemination is the identification of the three largest families of neural adhesion molecules, N-cadherins, N-CAMs, and integrins. Medulloblastomas express all three of these molecules[18–23] but available evidence suggests that not all three mediate leptomeningeal dissemination. N-cadherins and N-CAMs bind homophilically to form N-cadherin–N-cadherin and N-CAM–N-CAM bonds; these pairings may mediate cell–cell adhesion within the primary tumor.[15] However, the minimal expression of N-cadherins and N-CAMs in the meninges[24–26] suggests that these molecules are unlikely mediators of medulloblastoma adhesion to the meningeal surface. In contrast, integrins bind to a range of ECM proteins, many of which are expressed in the leptomeninges. These include collagens I,[11,27] III,[9,11,14] IV,[11,25,27] and VI;[28] procollagen III;[11] fibronectin;[11,26] tenascin;[29] laminin;[11,25,27,30] vitronectin;[31] and thrombospondin.[32] Integrins are also critical to the adhesion of other cancers that disseminate by surface spread rather than by infiltration, eg, ovarian cancer and mesothelioma.[33–36] We hypothesized a similar role for integrins and ECM proteins in leptomeningeal dissemination, ie, specific integrin–ECM protein pairings are necessary for the adhesion of medulloblastoma cells to the meninges.

The Akt and MAPK pathways are mediators of the survival and proliferation of many disseminated cancers but their role in disseminated medulloblastoma has not been investigated.[37] When growth factor is available, the two pathways are activated independently; Akt is activated by integrins following adhesion to ECM and MAPK is activated by soluble growth factors (Figure 1). However, in a growth factor poor environment, both are activated by integrins following adhesion.[37] Adhesion triggers integrin clustering and recruitment of adaptor molecules to focal adhesion complexes that are composed of a variety of protein kinases and adaptor molecules, including integrin-linked kinase (ILK), phosphatidylinositolphosphate-3, 4, 5 triphosphate, parvins, and paxillin; ILK activates Akt. In a growth factor deficient environment, growth factor cannot bind to RTK and, consequently, RTK to MAPK signaling does not occur. Instead, ILK engages a number of adaptor molecules, most notably PINCH-1 and Nck2. Nck2 subsequently activates MAPK through interaction with the cytoplasmic domain of RTK.[39,38] The leptomeninges are a growth factor poor environment;[40,41] as such, MAPK is unlikely to be activated by RTK signaling. We hypothesized that integrin adhesion to ECM in the process of leptomeningeal dissemination leads to activation of Akt and MAPK.

This study attempted to extend Rutka *et al*'s previously successful approach by combining their *in vitro* model[13] with recently developed immunoassays for integrin subunits and their ECM-binding partners. We produced adhesion of medulloblastoma cells to H4 ECM *in vitro* and used these novel immunoassays to identify $\alpha9\beta1$ integrin on the cell surface and tenascin in the ECM as the molecules that mediate this process. We then confirmed these findings *in vivo* through immunohistochemistry of medulloblastoma surgical specimens. Finally, we demonstrated that $\alpha9\beta1$ integrin-mediated adhesion is necessary for survival and proliferation of medulloblastoma cells in a growth factor poor environment and is associated with activation of the MAPK pathway.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA), EDTA, Triton-X100, fibronectin, laminin, and human insulin-like growth factor 1 (IGF-1) were purchased from Sigma (St Louis, MO, USA). Human tenascin-C was purchased from Millipore/Chemicon (Billerica, MA, USA). DMEM was purchased from ATCC (Manassas, VA, USA). All other cell culture products were purchased from Invitrogen/Gibco (Carlsbad, CA, USA). Monomeric and heterodimeric disintegrins were purified from lyophilized viper venoms as previously described.[42,43]

Antibodies

Adhesion and ELISA assays

Monoclonal antibodies to integrin subunits included anti-$\alpha1$ clones AJH10 and AEF3 (gifts from Dr P Wainreb, Biogen); anti-$\alpha2$ clone 26G8 (gift from Dr P Wainreb, Biogen); anti-$\alpha2$ clone P1E6 (Millipore/Chemicon); anti-$\alpha3$ clone p1B5 (Millipore/Chemicon); anti-$\alpha4$ clone HP2/1 (Beckman Coulter, Fullerton, CA, USA); anti-$\alpha5$ clone SAM1 (Beckman Coulter); anti-$\alpha6$ clone GoH3 (BD Pharmingen, San Diego, CA, USA); anti-$\alpha9/\beta1$ clone Y9A2 (Millipore/Chemicon); anti-$\alpha v\beta3$ clone LM609 (Millipore/Chemicon); anti-$\alpha v\beta5$ clone P1F6 (Millipore/Chemicon); anti-$\beta1$ clone Lia1/2 (Beckman Coulter); anti-$\beta2$ clone YFC118 (Millipore/Chemicon); anti-$\beta4$ clone 439-9B CBL545 (Millipore/Chemicon); anti-αL clone 38 (Millipore/Chemicon); anti-αM clone ICRF44 (Beckman Coulter). Polyclonal antibodies to fibronectin, laminin, tenascin, and vitronectin were purchased from Millipore/Chemicon as were monoclonal antibody clones, 23IIC3 and 4H12, to collagen type IV and IE5 to VCAM-1. Polyclonal antibody to TSP-1 was purchased from Calbiochem. Another polyclonal antibody to VWF was purchased from DacoCytomation (Carpentaria, CA, USA).

Immunohistochemistry

Rabbit polyclonal anti-$\alpha9$ integrin was produced by immunization with the cytoplasmic domain of the integrin and tested for specificity in western blots against SW480 cells transfected with $\alpha9$ integrin. Other antibodies included anti-$\beta1$ integrin (Abcam, Clone 4B7R); Tenascin (Abcam, Clone BC-24); Fibronectin (Transduction Laboratories, Lexington,

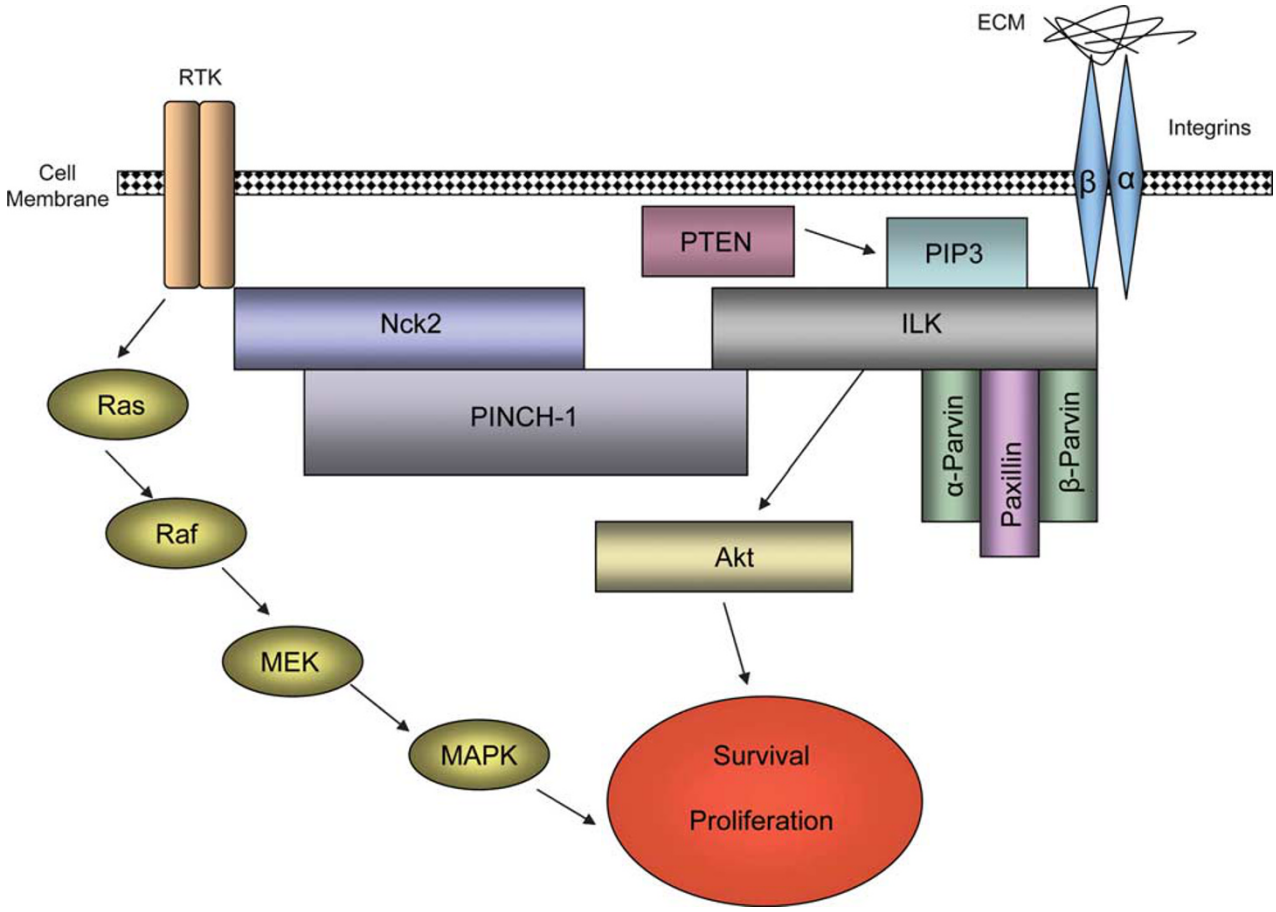

Figure 1 Integrin-RTK signaling. A coordinate signaling pathway from both integrins and receptor tyrosine kinases mediates cell survival and proliferation. Integrin-linked kinase (ILK) binds to the integrin β-subunit and is key to the assembly of a multiprotein complex; this multiprotein complex activates Akt. RTKs are linked via PINCH and Nck2 and initiate the Ras–MAPK pathway. (Modified from Cordes *et al*[38] with permission of the authors.)

KY, USA; Clone 10); Laminin (Sigma, rabbit polyclonal, (L9393); Collagen IV (Dako, Clone CIV22).

Western Blots

Antibodies to pan Akt (11E7); phospho- Ser473-Akt (193 H12), total extracellular signal-regulated kinase (Erk) 1/2 (p42/44) (9122) and phospho-Erk 1/2 (197G2) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-GRB2 (clone 81) was obtained from Transduction Laboratories.

Cells and Cell Culture

H4 glioma and D283 medulloblastoma cell lines were obtained from ATCC. The H4 glioma cell line was selected as the source of meningeal ECM. Although previous studies have demonstrated medulloblastoma cell adhesion to primary leptomeningeal cultures,[13] these cells are not widely available. Anatomy was the basis of selection of H4 cells as an alternative; glia line the pial brain surface and constitute a substantial proportion of the leptomeningeal cells.[44,45] The D283 medulloblastoma cell line was selected as the source of medulloblastoma cells because these cells originated in a tumor that had undergone leptomeningeal dissemination. All cell lines were maintained in ATCC DMEM with 10% fetal bovine serum. Cell counts were performed manually with a Brightline hematocytometer (Fisher Scientific, Pittsburgh, PA, USA). Cell viability was determined by trypan blue exclusion. Of note, in our hands the D283 cell line grows largely as nonadherent spheroids with a minority of the cells loosely attached to the plastic tissue culture flasks. To avoid introducing bias into the results by selecting one or the other of these populations the cells were harvested from the flasks with gentle scraping to insure that the adherent and nonadherent cells were equally represented. This was followed by trituration to dissociate the spheroids before counting. Experiments that required serum-free medium were performed in ATCC DMEM without additives. Petri dishes and 96-well plates were coated with fibronectin and laminin at 5 µg/ml. H4 ECM-coated surfaces were created with confluent cultures of H4 glioma cells grown on plastic surfaces. The cells surfaces were stripped with 0.1% Triton-X100 (30 min at RT) followed by 25 mM NH_4OH (1–2 min) and three rinses with

DH_20.[46] This preparation was used directly as H4 ECM for the experiments described. To achieve anchorage independence, sterile Petri dishes without tissue culture treated surfaces were coated with polyhema (Sigma; 12 mg/ml in 95% ETOH, 0.8 mg/cm^2).

Cell Adhesion Assays to Antibodies, Disintegrins, and Purified ECM Proteins

Antibodies and disintegrins were immobilized on 96-well microtiter plates (Falcon, Pittsburgh, PA, USA) by overnight incubation in phosphate-buffered saline (PBS) at 4°C. Antibodies were coated at 10 μg/ml, and disintegrins were coated at 20 μg/ml. Wells were blocked with 1% BSA at 37°C for 1 h. Cells were labeled by incubation with 12.5–25 μM 5-chloromethylfluorescein diacetate (CMFDA) in Hank's balanced salt solution (HBSS) containing 1% BSA for 15 min. Unbound label was removed by washing in the same buffer. (For experiments requiring calcium-free medium, calcium-free HBSS supplemented with 5 mM EDTA was used.) Labeled cells were counted by hemocytometer and diluted to a concentration of 10^6 cells/ml. A portion of 100 μl (10^5 cells) was then added to each well and plates were incubated at 37°C for 30 min. Unbound cells were removed by washing, and bound cells were lysed by the addition of 0.5% Triton X-100. A standard curve was prepared using known concentrations of labeled cells. Plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore) with a 485-nm excitation filter and a 530-nm emission filter. For inhibition studies, an identical protocol was followed, except that the cells were incubated with antibodies (10 μg/ml) for 1 h at 37°C before the addition of CMFDA. The percentage inhibition of binding was calculated by comparison with the fluorescence values obtained from control samples without added antibody. For the experiments with adhesion to purified ECM proteins, human tenascin-C and collagen IV were coated at 1 ug/cm^2 for 1 h at 37°C. Assays were conducted with the same technique as above except that wells were not blocked with BSA and plates were incubated at 37°C for 1.5–2 h to achieve adhesion.

Elisa Assay for ECM Protein Content of H4 Matrix

96-Well plates coated with H4 ECM were blocked with 1% BSA in Tris-buffered saline (TBS)/Tween 20 (TBST) buffer at 37°C for 1 h. Either rabbit polyclonal (1 μg/ml) or mouse monoclonal (5 μg/ml) antibodies were added in TBST, and the plate was incubated for 2 h at 37°C. Wells were washed four times with TBST. Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse (Sigma) was diluted 1:3000 in TBST plus 1% BSA and incubated for 1 h with the plate. After washing four times with TBST, Sigma 104 alkaline phosphatase substrate at 1 mg/ml in TBS, pH 9.0, was added to each well. Color development was monitored at 405 nm.

Immunocytochemistry

D283 cells were passaged onto H4 ECM-coated microscope slide culture chambers (Labtech; Nalge NUNC Intl, Rochester, NY, USA) and incubated for 24–48 h at 37°C under standard cell culture conditions. The resulting cultures were fixed in cold (4°C) acetone for 1–3 min and then washed three times in PBS. Slides were blocked in 10% normal horse serum in PBS 0.1% BSA for 30 min. Incubation in primary antibodies (diluted in 0.1% PBS-BSA) was at 37°C for 1 h. Slides were washed three times in PBS and incubated in secondary fluorescent antibody (1:500; Vector Labs, Burlingame, CA, USA) in 0.1% PBS-BSA for 1 h. in dark. The resulting slides were washed three times in PBS and mounted with water soluble medium (Vectashield; Vector) before fluorescence microscopy.

Immunohistochemistry

Antibodies to the α9 and β1 integrin subunits and the ECM proteins tenascin, fibronectin, laminin, and collagen IV were used to stain normal adult human cerebellum and seven surgical specimens of cerebellar medulloblastoma with leptomeningeal extension obtained from the UHN Pathology Archive. The medulloblastoma specimens selected contained both the primary tumor and the interface between cerebellum and leptomeningeal tumor. The histologic subtypes of these tumors were classic[4] and nodular/desmoplastic.[3] Paraffin-embedded sections (4–6 μm thick) were dewaxed in xylene and rehydrated in ascending alcohols. Following antigen retrieval with Na citrate (10 mM, pH 6.0 heating to 120°C for 2 min in decloaking chamber (Biocare, Concord, CA, USA)) or pepsin (1% in 0.01 N HCl, pH 2.0 for 15 min at 37°C), sections were incubated with primary antibody at room temperature. In preparation for anti-α9 integrin staining, tissue was pretreated with pepsin; primary antibody was applied at 1/4000 dilution for 1 h. For anti-β1 integrin staining, tissue was pretreated with pepsin; primary antibody was applied at 1/100 for 1 h. For antitenascin staining, tissue was pretreated with pepsin; primary antibody was applied at 1/4000 dilution overnight. For antifibronectin staining, tissue was pretreated with pepsin; primary antibody was applied at 1/1000 dilution for 1 h. For antilaminin staining, tissue was with pepsin; primary antibody was applied at 1/100 dilution for 1 h. For anti-collagen IV staining, tissue was pretreated with citrate; primary antibody was applied at 1/100 dilution for 1 h. Primary antibodies were subsequently amplified with secondary antibody and then with avidin–biotin complex coupled to horseradish peroxidase. Chromagen was developed with diaminobenzidine. Following light counterstaining with hematoxylin, sections were dehydrated and coverslipped with permount.

Western Blots

Cells were lysed in 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride,

0.2 mmol/l sodium orthovanadate. Aliquots of 50 μg of protein extracts were separated in a 4% to 15% gradient SDS–PAGE (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. The resulting blots were blocked in 5% nonfat milk and probed with anti-Erk 1/2 and anti—phospho-Erk as well as 1/2 anti-Akt and anti-phospho-Akt (Ser473). In addition, blots were probed for GRB2 as a loading control. Western blots were developed with the ECL system (Perkin Elmer) and archived onto KODAK X-OMAT film. Films were scanned with Alphaimager HP and analyzed with GelFox (Alphainnotech, San Leandro, CA, USA).

Charts and Statistical Measures

The between-group differences from cell adhesion assays, ELISA assays, and western blots were initially tested for significance via single-factor ANOVA. One-tailed *t*-tests were subsequently performed to assess differences between groups; unequal variance across groups was assumed. $P<0.05$ was considered statistically significant for all comparisons. All analyses and graphs were performed with Microsoft Excel. Asterisks above bars indicate significant differences. Error bars on graphs represent the standard error of the mean. Each datapoint represents the average of the data obtained from three to five experiments. In each experiment, there were either duplicate or triplicate wells/conditions. As such, each datapoint represents the average of 6–15 independent measures.

RESULTS

Medulloblastoma Cells Adhere to H4 Glioma Matrix

Figure 2 provides phase-contrast images of D283 cells grown for 24 h on tissue culture plastic, laminin, fibronectin, and H4 matrix. In comparison to the loose adherence normally shown by D283 cells on tissue culture plastic (a) no qualitative increase in surface attachment was observed with laminin (b); and a modest increase in attachment was observed in the context of fibronectin (c). In contrast, D283 cells demonstrated significant adhesion to the H4 matrix (d); the spreading expected to follow surface attachment in epithelial cells was also observed.

Adhesion to H4 Glioma Matrix is Dependent on Extracellular Ca++ Concentration

When D283 cells were incubated in H4 matrix-coated wells in the presence of Ca++, adhesion of 15 000 cells per well was observed. In contrast, when these cells were incubated in the absence of Ca++, adhesion of only 1000 cells per well was observed. This 15-fold decrement in adhesion was significant ($P<0.02$; Figure 3). This suggests that adhesion is mediated by cationic dependent molecular pairings such as those between integrins and ECM proteins; cationic independent pairings would predominate if N-CAMs accounted for the adhesion of D283 cells to H4 matrix.[16,17]

Medulloblastoma Cells Express α9 and β1 Integrin Subunits

The level of integrin expression by D283 cells was determined by semiquantitative assays of D283 cell adhesion to anti-integrin antibodies immobilized on 96-well plates (Figure 4). The 15 anti-integrin antibodies included 9 to α-subunits (1, 2, 3, 4, 5, 6, 9, L, M), 3 to β-subunits,[1,2,4] and 2 to heterodimers αvβ3 and αvβ5. ANOVA demonstrated significant differences among α-subunit group measures ($P<1.1\times10^{-11}$). Subsequent *t*-tests demonstrated that expression of the α9-subunit was significantly greater than expression of the eight other α-subunits and the αv-heterodimer ($P<0.03$ for all *t*-tests performed). Similarly, ANOVA demonstrated significant differences among β-subunit group measures ($P<2.0\times10^{-11}$) and subsequent *t*-tests demonstrated that expression of the β1-subunit was significantly greater than expression of the two other β-subunits and the αvβ3 and αvβ5 heterodimers ($P<4.6\times10^{-5}$ for all *t*-tests performed).

Antibodies and Disintegrins against α9 and β1 Integrins Inhibit Adhesion of Medulloblastoma Cells to H4 Matrix

Eight of the antibodies used in the adhesion assays above (α1, α2, α3, α9, β1, β2, β4, αvβ3) were added to aliquots of D283 cells at a concentration (10 μg/ml) sufficient to complex with the majority of integrin subunits expressed on the cell surfaces. These cells were then placed in wells coated with H4 ECM and their adhesion to that surface assayed (Figure 5a). Antibody to the α9-subunit inhibited 99% of adhesion and antibody to the β1-subunit inhibited 100% of adhesion to the H4 matrix. Again, ANOVA demonstrated significant differences among the eight subunit groups ($P<4.8\times10^{-14}$). *t*-Tests demonstrated significantly greater inhibition for α9 and β1 than for all the other subunits (α1, α2, α3, β2, β4, αvβ3; $P<0.005$ for all *t*-tests performed).

In a parallel experiment, disintegrin peptides, snake venom proteins with selective abilities to complex with integrins, were employed with the same assay design (Figure 5b). VLO5, which binds to the α9β1 integrin heterodimer,[43] inhibited 95% of adhesion. Echistatin, which does not block the α9 integrin subunit but is a potent antagonist of αIIbβ3, avβ3, and α5β1,[42,47] inhibited only 25% of adhesion. The extent of inhibition by VLO5 was significantly different from the extent of inhibition by Echistatin ($P<0.04$).

Tenascin is the Predominant H4 ECM Protein

The α9β1 heterodimer is known to bind preferentially to tenascin-C or VCAM-1 and the β1-subunit interacts with a variety of ECM proteins including fibronectin, laminin, vitronectin thrombospondin-1, collagen, and Von Willebrand Factor.[42,47] Antibodies to these proteins were used in an ELISA format to assay H4 matrix-coated wells (Figure 6). The signal for antitenascin was more than twice as intense as that of the next largest signal (collagen IV). Single-factor ANOVA demonstrated significant differences among signal

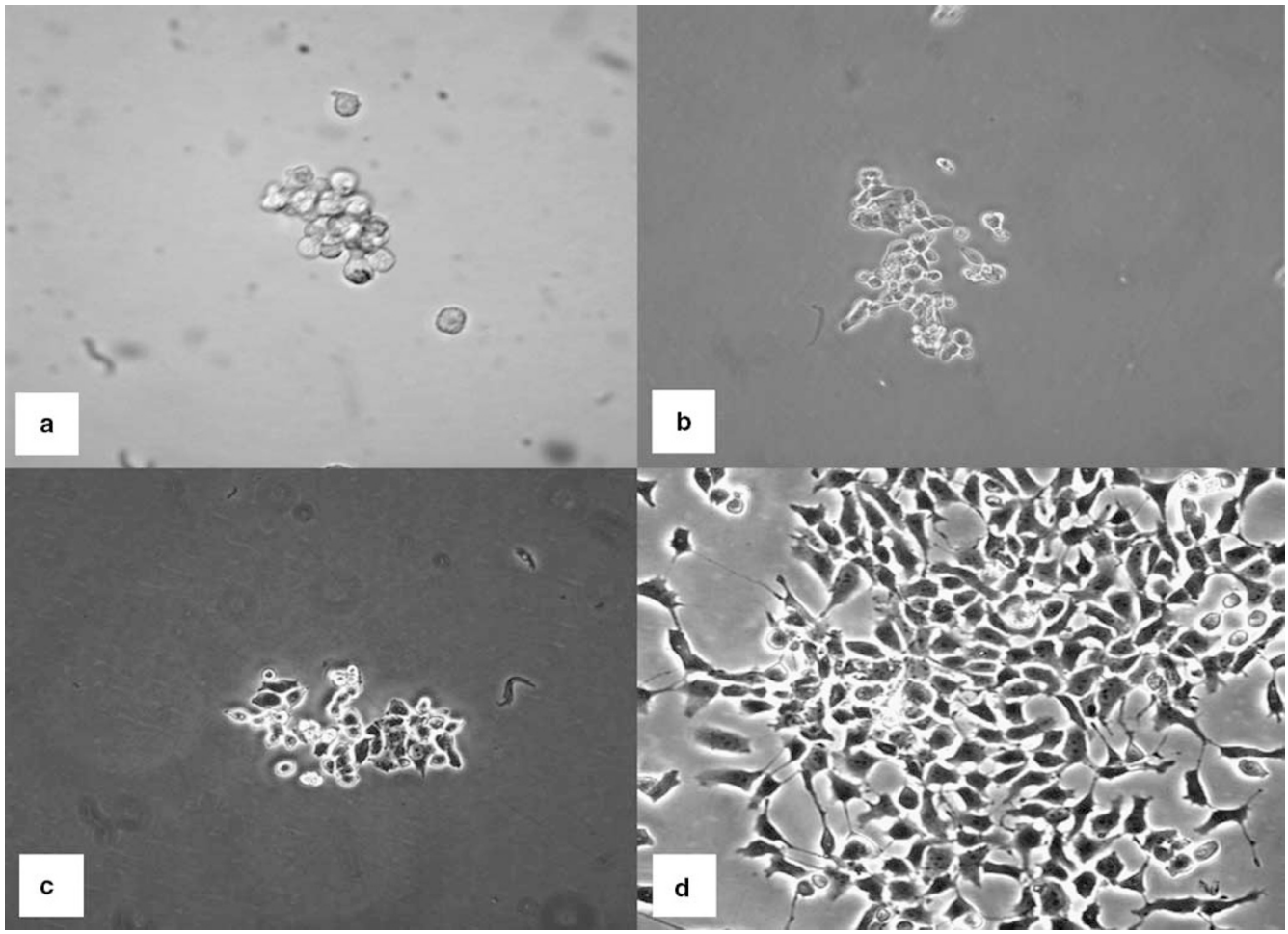

Figure 2 D283 medulloblastoma cells are more adherent to H4 matrix than tissue culture plastic, laminin, or fibronectin. Phase-contrast images demonstrate the minimal adhesion characteristic of D283 cells grown on tissue culture plastic (**a**); the degree adhesion of cells grown on laminin was similar to that of the control (**b**). Modest adhesion to fibronectin was observed (**c**). D283 cells adhered to the H4 matrix; the spreading expected to follow surface attachment in epithelial cells was also observed (**d**).

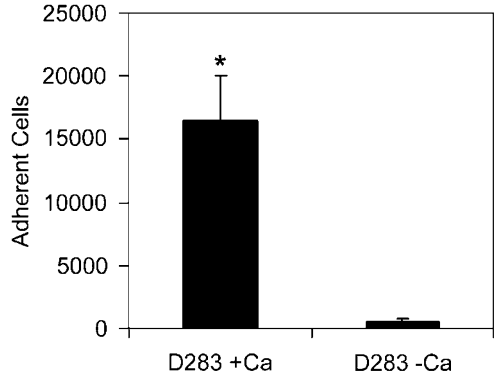

Figure 3 Adhesion of D283 cells to H4 matrix is calcium dependent. In the presence of Ca++, 15,000 cells adhered per well. In the absence of Ca++, a fifteenfold decrement in the binding of D283 cells to H4 matrix was observed (*$P<0.02$).

intensities ($P<3.4\times10^{-10}$), and subsequent *t*-tests demonstrated significant difference between the tenascin signal and the intensity of all other signals ($P<0.006$ for all *t*-tests performed).

Medulloblastoma Cells' Adhesion to Purified Tenascin is Dependent on α9 And β1 Integrins

As tenascin and collagen IV are the two most abundant proteins in the H4 matrix, we compared D283 cell adhesion to isolated, purified tenascin-C, and collagen IV. 8% of cells adhered to tenascin-C, 4% to collagen IV, and 2% to the control-untreated tissue culture plastic. (See Figure 7a.) Single-factor ANOVA demonstrated significant differences among these percentages ($P<3\times10^{-4}$). Notably, the percent of D283 cells that adhered to tenascin-C was greater than the percent that adhered to both collagen IV and control ($P<0.049$).

In parallel assays, D283 cells were incubated in anti-α9 or anti-β1 integrin and then were placed in wells coated with tenascin-C or collagen IV. Antibody to α9 inhibited 65% of adhesion to tenascin-C but only 25% of adhesion to collagen IV. Antibody to β1 inhibited 60% of adhesion to tenascin-C but only 32% of adhesion to collagen IV. *t*-Tests demonstrated significantly greater α9 and β1 inhibition of adhesion to tenascin-C than to collagen IV ($P<2\times10^{-4}$ and 0.03 respectively; asterisks in Figure 7b).

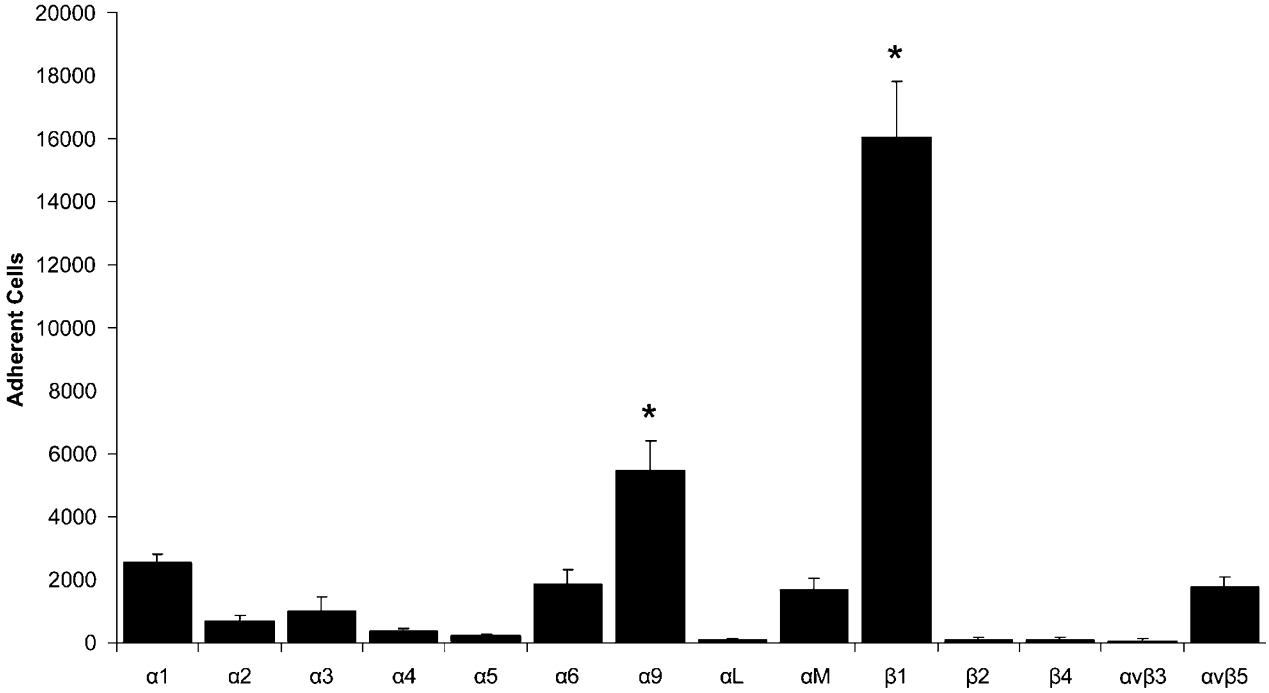

Figure 4 D283 cells preferentially express $\alpha 9$ and $\beta 1$ integrin subunits. $\alpha 9$ expression was greater than expression of the other eight α-subunits (1, 2, 3, 4, 5, 6, L, M) ($^*P < 0.03$ for all *t*-tests performed). $\beta 1$ expression was greater than expression of the two other β-subunits[1,2,4] and the $\alpha v\beta 3$ and $\alpha v\beta 5$ heterodimers ($^*P < 4.6 \times 10^{-5}$ for all *t*-tests performed).

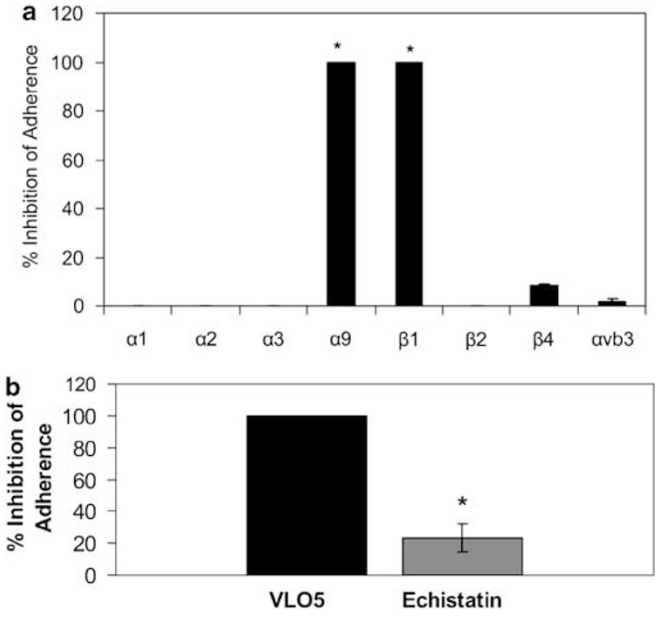

Figure 5 Inhibition of adhesion by antibodies and disintegrins against the $\alpha 9$ and $\beta 1$ integrin subunits. (**a**) Antibody to the $\alpha 9$-subunit inhibited 99% of adhesion and antibody to the $\beta 1$-subunit inhibited 100% of adhesion to the H4 matrix ($^*P < 0.005$ for all *t*-tests performed). (**b**) VLO5 inhibited 95% of adhesion. Echistatin inhibited only 25% of adhesion ($^*P < 0.04$).

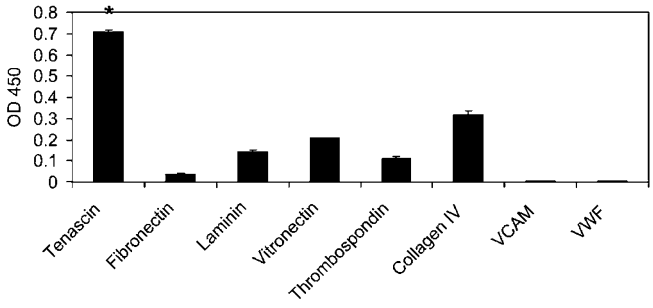

Figure 6 Tenascin is the dominate protein expressed in H4 ECM. The signal for antitenascin was more than twice as intense as that of the next largest signal (collagen IV) ($^*P < 0.006$ for all *t*-tests performed).

$\alpha 9$ and $\beta 1$ Integrin are Localized to D283 Cells by Immunocytochemistry

D283 cells grown on the H4 matrix were labeled immunocytochemically with both anti-$\alpha 9$ integrin and FITC-labeled VLO5. Both ligands demonstrated pronounced cell surface and cytoplasmic labeling (Figure 8a and b). Cells stained in parallel with either antibody to $\beta 2$ integrin or FITC-labeled bitisatin (a disintegrin that does not bind either the $\alpha 9$ or $\beta 1$ integrin)[42] did not label (data not shown).

Immunohistochemistry of Surgical Specimens Demonstrates Increased $\alpha 9$ and $\beta 1$ Integrin and Tenascin Reactivity in Leptomeningeal Medulloblastoma

In normal cerebellum (Figure 9a) antibodies to $\alpha 9$ integrin, $\beta 1$ integrin, tenascin, laminin, and collagen IV demonstrated minimal staining. The endothelium was sparsely labeled by $\alpha 9$, $\beta 1$, and laminin; the vascular media also stained weakly

with tenascin and collagen IV (black arrows). Focal leptomeningeal staining for α9 integrin, β1 integrin, tenascin, and laminin was observed (red arrows). There was no staining for fibronectin; this is consistent with data from previous studies of paraffin-embedded aldehyde-fixed normal CNS tissues.[48–52] However, our positive controls for fibronectin including human lung, colon, and kidney did demonstrate staining of the basement membrane side of the vasculature (data not shown).

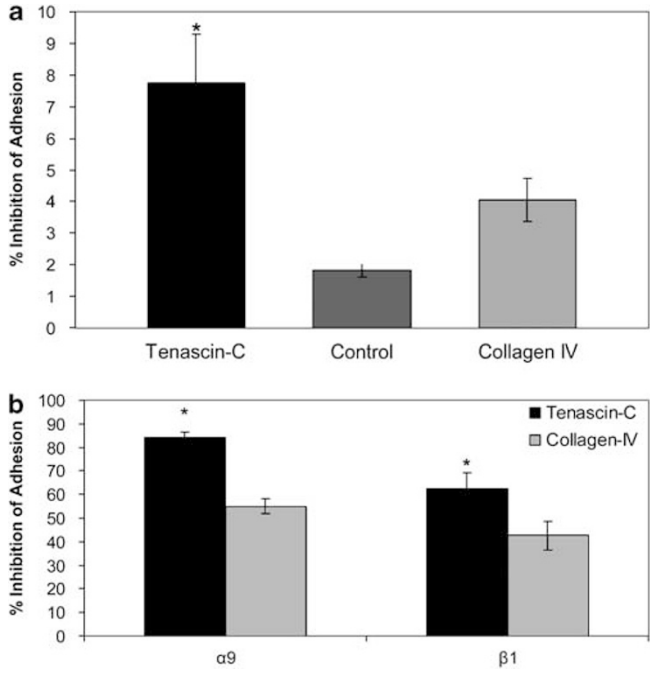

Figure 7 Tenascin is the preferential substrate for α9β1 D283 adhesion. (**a**) More D283 cells adhere to tenascin-C than to control uncoated wells or to collagen IV (*$P<0.049$). (**b**) Antibodies to both α9 and β1 integrin inhibit the adhesion of D283 cells to tenascin more than the adhesion to collagen IV (*$P<2\times10^{-4}$ and 0.03, respectively).

Primary medulloblastomas (Figure 9b) also demonstrated low levels of reactivity for α9 and β1 integrin. In the seven tumors, antibody to the α9 integrin labeled the cell surface of two (one classic, one nodular) and anti-β1 integrin labeled six (three classic, three nodular). Discrete tenascin reactivity was observed between the cells of one nodular tumor. Although the source of the tenascin staining is not known, it may represent reactive astrocytic processes that are often present in medulloblastomas[53] and are known to express tenascin.[54] Vessels within six of these seven tumors demonstrated reactivity for fibronectin (red arrows). Antilaminin demonstrated focal positivity in two tumors (one classic, one nodular) as well positive, more intense vascular staining than the control cerebellum (red arrow). There was no collagen IV staining of tumors. As with fibronectin and laminin, occasional vascular staining was observed (red arrow). The increased vascular labeling for fibronectin, laminin, and collagen IV may reflect the diminution of blood brain barrier integrity within the tumor that allows subendothelial deposition of ECM proteins.[51,52] In the nodular medulloblastomas, there was no preferential immunoreactivity of the nodular or internodular regions with any of the antibodies.

Most leptomeningeal extensions of tumor (Figure 9c) demonstrated increased reactivity for α9, β1, and tenascin.

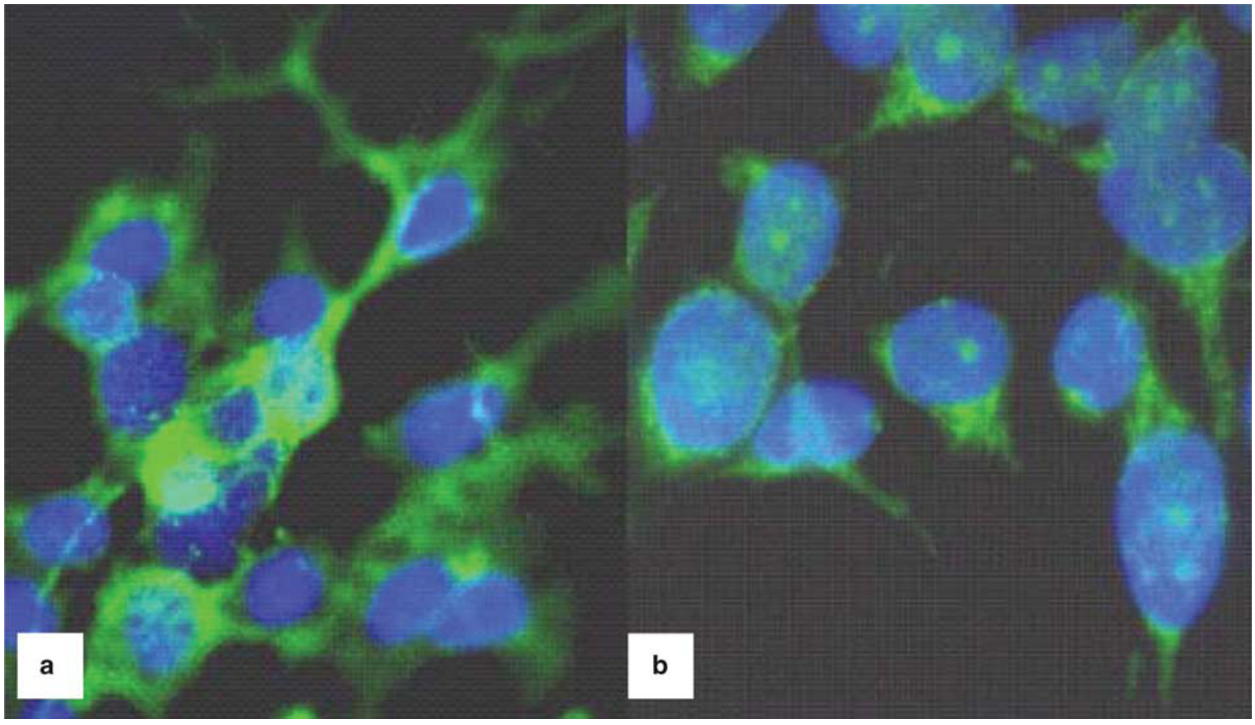

Figure 8 Immunocytochemistry of α9 and β1 integrins in D283 cells. Both cell surface and cytoplasm of D283 cells labeled with (**a**) anti-α9 integrin and (**b**) VLO5.

The pattern of expression was similar across tumors, ie, α9 and β1 expression was greatest at the periphery of the leptomeningeal tumor implants (black arrows) but tenascin was concentrated in the deeper parts of the leptomeningeal tumor (red arrows). This pattern characterized the two tumors originally positive for α9, five of the six originally positive for β1, and all six of those originally negative for tenascin. There was no increased reactivity for fibronectin, laminin, or collagen IV in any extension although occasional laminin positive vessels were seen (black arrows).

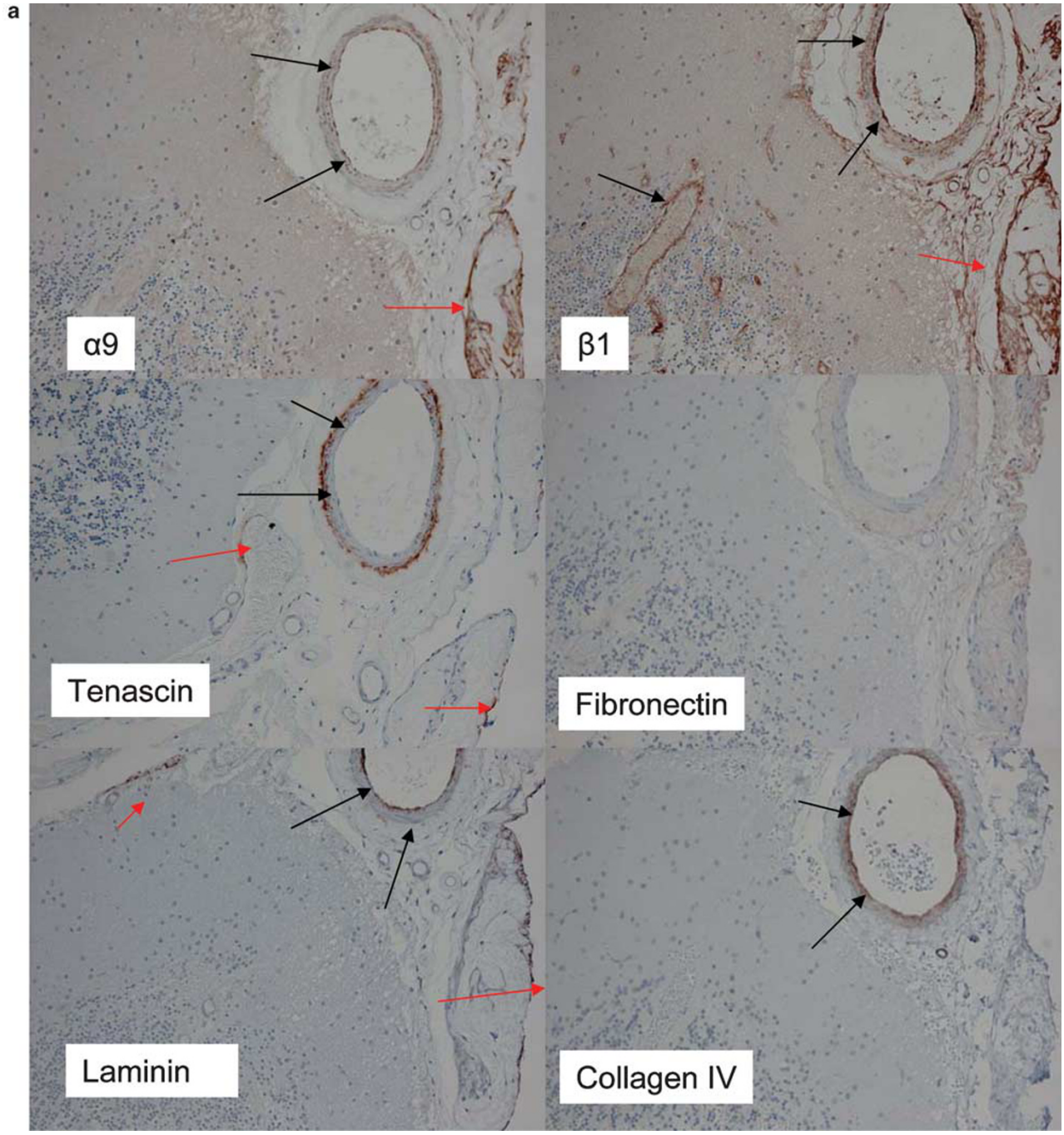

Figure 9 Immunohistochemistry: increased expression of α9, β1, and tenascin in leptomeningeal implants. (**a**) In the normal cerebellum, staining for α9, β1, tenascin, laminin, and collagen IV was limited to sparse reactivity in vessels and meninges (black and red arrows, respectively). (All original images × 200 magnification.) (**b**) In primary medulloblastoma cell surface staining for α9 and β1 was present at low levels. Tenascin reactivity was observed between the cells of one tumor. There was focal laminin positivity in two tumors. Vascular staining for fibronectin, laminin, and collagen IV was observed (red arrows). (All original images × 400 magnification.) (**c**) In leptomeningeal implants, staining for α9, β1 (periphery, black arrows), and tenascin (interface of tumor and cerebellum red arrows) was considerable. Occasional vessels in the implants stained for laminin (black arrows). (All original images × 200 magnification.)

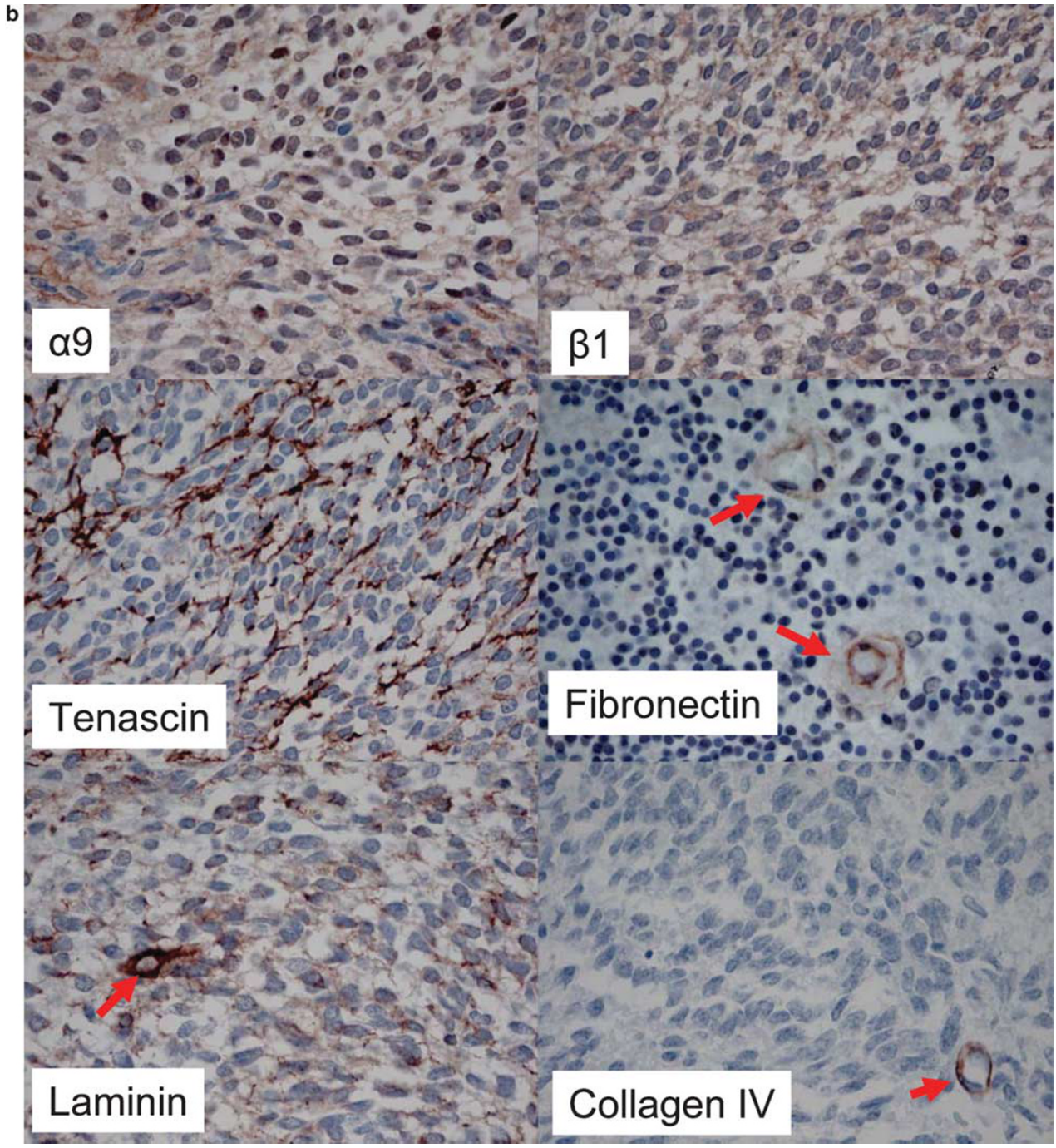

Figure 9 Continued.

Matrix Adhesion is Necessary for D283 Cell Survival in Serum-Free Conditions

To test the effect of surface adhesion on the survival of leptomeningeal tumor, 10^5 D283 medulloblastoma cells were plated in wells with serum-free medium (Figure 10). The viability of cells plated on H4 matrix was only 55% at 24 h and 40% at 48 h but was restored to 80% of the input value by 72 h due to proliferation. In the absence of H4 matrix, 80% of cells were nonviable after 72 h. Addition of blocking antibody to either $\alpha 9$ or $\beta 1$ integrin to cells cultured in H4 matrix-coated wells produced survival curves similar to those observed for the nonadherent cells. Microscopic observation of the wells to which antibody was added revealed that cells failed to adhere to substrate; this suggests that surface adhesion and cell survival are closely linked in serum-free cultures.

The decreased survival on days 1 and 2 among D283 cells on ECM is consistent with the absence of normal growth

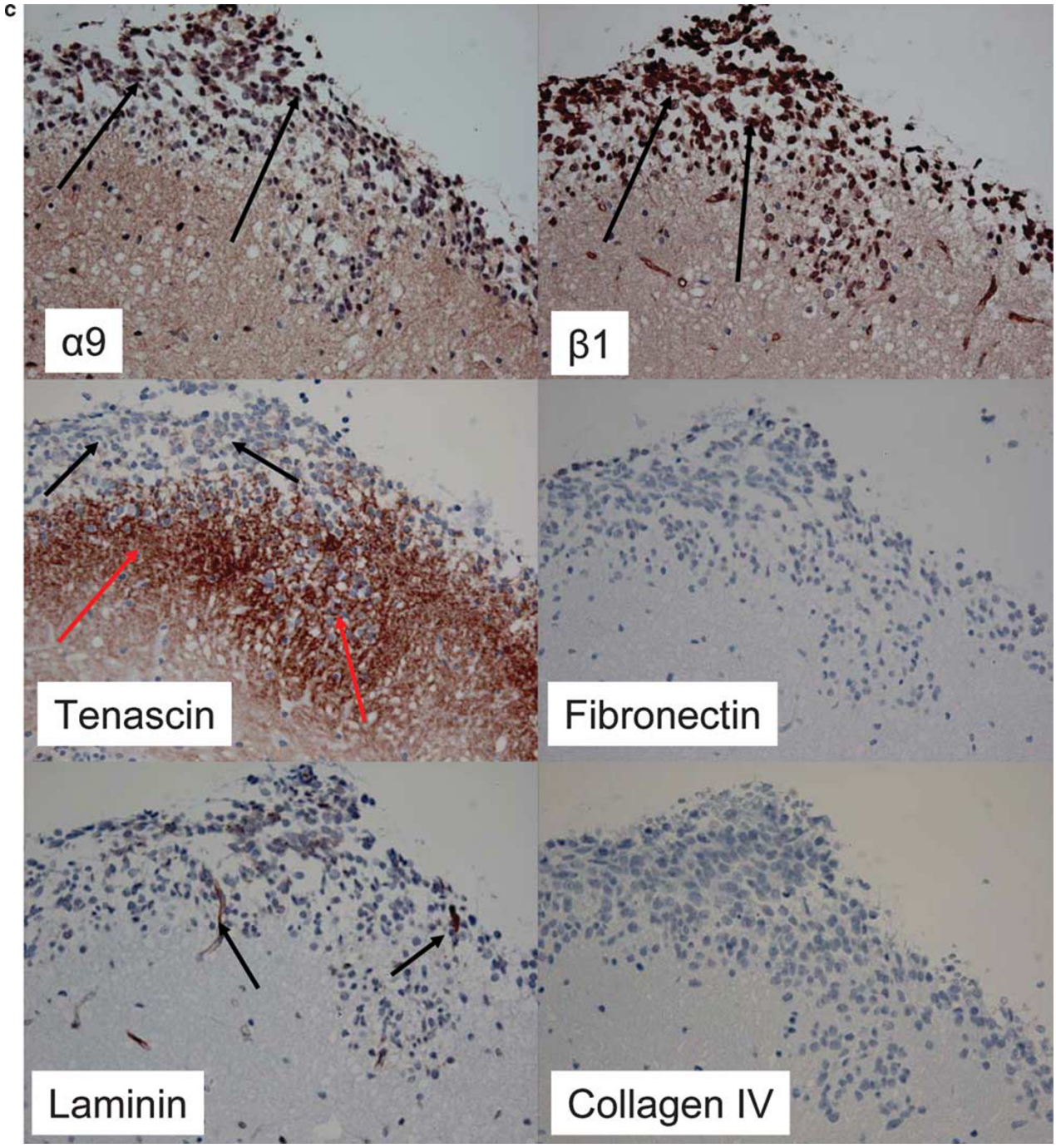

Figure 9 Continued.

conditions for these cells, ie, the absence of nonadherent spheroids. Adhesion to matrix eliminates the spheroid formation and, as a result, is likely to decrease cell–cell signaling; as such, additional time may be required for cells to develop alternate signaling pathways that promote proliferation.

Effects of Matrix Adhesion on Activation of Akt and MAPK

D283 medulloblastoma cells cultured in serum-free DMEM (ATCC) for 18–24 h in surface nonadherent conditions (polyhema-coated plates) were transferred to H4-coated plates. Parallel cultures in dishes coated with polyhema to block adhesion but treated with IGF1 (50 ng/ml for 0.5 h) served as positive controls; cells that were not transferred served as negative controls. Plates were harvested at 0.5, 1, 2, and 4 h. Proteins were extracted (50 μg per lane electrophoresed on 7.5% = acrylamide gels) and blotted to nylon membranes. The membranes were sequentially probed with antibodies to phospho-Erk1/2 and total Erk1/2. Membranes were reprobed with antibodies to phospho-Akt (Ser 473) and

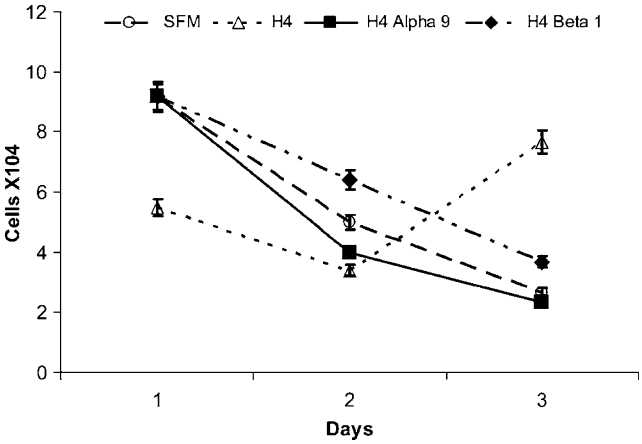

Figure 10 D283 cells are rescued from death by adhesion to H4 matrix. D283 cells cultured in serum-free medium (SFM) in the presence of H4 matrix survived and proliferated. Absence of matrix or blockade by $\alpha 9$ or $\beta 1$ integrin eliminated the survival benefit.

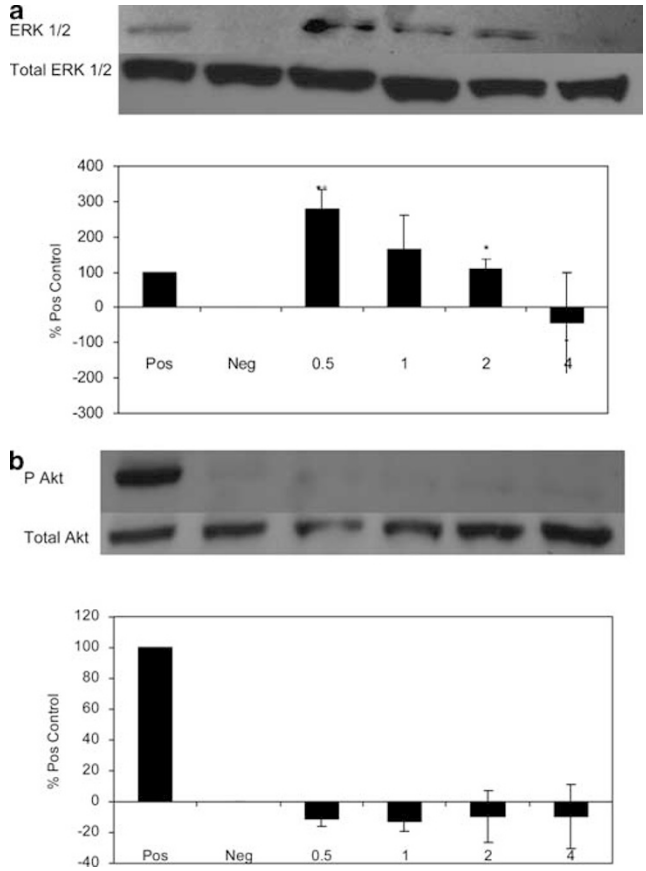

Figure 11 MAPK is activated following matrix adhesion of D283 medulloblastoma cells. Adhesion of D283 cells to H4 matrix resulted in threefold increase in MAPK phosphorylation (**$P<0.02$). At 2 hours, MAPK phosphorylation was still significantly greater than the controls (*$P<0.009$) (**a**). It did not activate Akt (**b**).

total Akt. Single-factor ANOVA demonstrated significant differences in levels of Erk1/2 phosphorylation across groups ($P<0.05$; Figure 11a). Phospho-Erk1/2 demonstrated a threefold increase relative to the positive control at 0.5 h ($P<0.02$). Phospho-Erk1/2 demonstrated a significant increase relative to the negative control at 0.5 and 2 h following transfer to the adhesive matrix ($P<0.007$ and $P<0.009$, respectively) but was not significantly different at 4 h ($P<0.04$) Single-factor ANOVA demonstrated no significant differences in Akt phosphorylation across groups ($P<0.81$; Figure 11b).

DISCUSSION

Both the *in vitro* and the *in vivo* data from this study suggest that adhesion of D283 medulloblastoma cells to the meninges is mediated by the interaction of cell surface $\alpha 9$ and $\beta 1$ integrins with ECM tenascin. *In vitro*, $\alpha 9$ and $\beta 1$ were the predominant integrin subunits expressed by the D283 cells and tenascin was the dominant protein expressed by the H4 matrix. Consistent with cationic dependent molecular pairings known to characterize integrins and ECM proteins, adhesion of the D283 cells to the H4 ECM was Ca++ concentration dependent. Blockade of the $\alpha 9$ and $\beta 1$ integrins eliminated binding both to the H4 ECM and to tenascin-C. The percent of D283 cells that adhered to tenascin-C was significantly greater than the percent that adhered to collagen IV, the second most abundant protein in the H4 matrix. *In vivo*, immunohistochemistry of surgical specimens demonstrated increased expression of $\alpha 9$ and $\beta 1$ integrins as well as tenascin at the junction of medulloblastoma and meninges.

The data from this study also suggest that, in a growth factor deficient environment, $\alpha 9$ and $\beta 1$ adhesion to ECM is critical to medulloblastoma cell survival and proliferation. D283 cells survived and proliferated in serum-free conditions following adhesion to ECM. Blockade of adhesion with antibodies to $\alpha 9$ and $\beta 1$ integrins eliminated these survival and proliferation advantages. D283 cell adhesion, in turn, stimulated transient phosphorylation of MAPK; phosphorylation was not observed in the absence of adhesion. These MAPK activation data are similar to results of previous studies of integrin-mediated cell adhesion to substrate.[55,56] In our study, 72 h elapsed before the proliferative advantage was observed. This interval may be explained by the known nuclear translocation of ERK1 and ERK2 that follows MAPK phosphorylation. This, in turn, phosphorylates transcription factors such as ELK1 and stimulates expression of cyclin D1, inducing cell cycling.[57,58]

Our finding of preferential MAPK activation following integrin-mediated adhesion is distinct from the marked activation of both the Akt and MAPK pathways that characterizes signaling following adhesion with the majority of integrin heterodimers.[37-39] Notably, the differential activation pattern we observed also describes the differential signaling of $\alpha 4\beta 1$, the integrin with greatest structural similarity to $\alpha 9\beta 1$;[43,59,60] this suggests the hypothesis that the similar signaling patterns of $\alpha 9\beta 1$ and $\alpha 4\beta 1$ are the result of their structural similarities. It also suggests that the MAPK pathway may play a greater role than the Akt pathway in the

survival and proliferation of other cancer cells that express predominantly the $\alpha 9\beta 1$ or $\alpha 4\beta 1$ integrins.

The data from this study support the use of H4 glioma cells as a matrix source for the study of leptomeningeal dissemination. Our demonstration of adhesion, survival, and proliferation with H4 glioma cells as the substrate was similar to the results of the previous study that employed primary leptomeningeal cells as the substrate.[13] In addition, the similarities in results of the two studies parallel similarities in the ECM protein profiles of the two substrates, ie, expression of fibronectin, laminin, and collagen type IV.[9,11,14] The significant difference in the results of the two studies was our identification of tenascin as the dominant protein in the adhesion process; assays for tenascin were not widely available when the earlier investigation was conducted and, as such, the roles of tenascin in adhesion to the different substrates cannot be compared. The expression of tenascin in the *in vivo* meninges adjacent to disseminated medulloblastoma is consistent with the viability of H4 glioma cells as an appropriate matrix source for investigations of leptomeningeal dissemination. The additional protein profile similarities of H4 glioma cells and *in vivo* meninges, ie, expression of fibronectin, laminin, vitronectin, thrombospondin, and collagen type IV but no detectable VCAM or von Willebrand factor[25–27,30–32,61,62] (also our immunohistochemical data) provide additional evidence for the appropriateness of H4 glioma cells as a substrate. Finally, the similarities in results between our study and the previous study[13] are consistent with the similarities in the anatomical roles of the H4 glioma and primary leptomeningeal cells. Both types of cells are observed in the meninges; the leptomeningeal cells comprise the arachnoidal trabeculae and the glial cells form the pia limitans.[44,45]

In summary, the results of this study provide preliminary evidence that coculture of D283 medulloblastoma cells with H4 glioma matrix results in adhesion of D283 cells to the ECM, and improved survival relative to nonadherent cells. Our results *in vitro* and *in vivo* also suggest that the expression of $\alpha 9\beta 1$ by medulloblastoma cells and tenascin in the ECM is necessary for adhesion. Additional *in vitro* results suggest that this adhesion activates the MAPK pathway and is necessary for both cell survival and proliferation.

ACKNOWLEDGEMENT

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