

# Glioblastoma Expression of Vitronectin and the $\alpha v\beta 3$ Integrin

## Adhesion Mechanism for Transformed Glial Cells

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### Abstract

Glioblastoma multiforme, the most malignant astroglial-derived tumor, grows as an adherent mass and locally invades normal brain. An examination of adult cerebral glioblastoma biopsy material for the expression of adhesive proteins that might potentiate adhesion and invasion demonstrated tumor cell-associated vitronectin (5/5). In contrast, vitronectin was not detected associated with glial cells in low grade astroglial tumors (0/4), reactive astrogliosis (0/4), or in normal adult cortex and cerebral white matter (0/5). Also, a wide variety of other adhesive ligands were absent from the glioblastoma tumor parenchyma. The  $\alpha v\beta 3$  integrin was the only vitronectin receptor identified in glioblastoma tumors in situ, and was also not expressed on low grade astroglial-derived tumors, reactive astrogliosis, or on glia or neurons in normal adult cortex and cerebral white matter. In a cell attachment assay, cultured glioblastoma cells attached to the parenchyma of glioblastoma tumor cryostat sections at the sites of vitronectin expression, but failed to attach to normal brain. This adhesion was inhibited by antibodies directed against vitronectin, the  $\alpha v\beta 3$  integrin, and with an Arg-Gly-Asp-containing peptide. These data provide evidence for a cell adhesion mechanism in glioblastoma tumors that might potentiate glioblastoma cell invasion of normal brain. (*J. Clin. Invest.* 1991. 88:1924-1932.) Key words: glioblastoma • invasion • vitronectin •  $\alpha v\beta 3$  integrin

### Introduction

Tumor invasion is thought to involve adhesive interactions between tumor cells and the host extracellular matrix (1, 2). Thus, one approach to understanding tumor cell invasion is to identify the matrix proteins and their receptors within the tumor parenchyma. Glioblastoma multiforme (GBM),<sup>1</sup> the highest grade astroglial-derived tumor, grows in an adhesive manner in culture (3), suggesting the presence of cell adhesion receptors. However, little is known about the extracellular adhesive ligands or cell adhesion receptors present in situ

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1. Abbreviations used in this paper: GBM, glioblastoma multiforme; IHC, immunohistochemistry; NRS, normal rabbit serum; VN, vitronectin; VNR, vitronectin receptor.

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within these tumors. GBM tumors are derived from glial cells, and in normal adult brain these cells and the surrounding extracellular matrix (neuropil) are devoid of most, if not all, previously characterized extracellular matrix adhesive proteins (4-8).

In an effort to understand the adhesive interactions within GBM tumors, we examined GBM cryostat sections for the expression of a variety of extracellular matrix adhesive proteins. Among the proteins examined, within the parenchyma of GBM tumors, only vitronectin was detected. Vitronectin is an adhesive protein normally found in plasma, and has been shown to promote the adhesion of a wide variety of cell types in vitro (9-13). Vitronectin has been identified in the embryonic chick retina, where it serves to promote neurite extension (14); however, in the adult mammal no adhesive function for vitronectin in situ has been identified. Vitronectin has several other functional domains, such as the collagen- and heparin-binding domains (13), that are likely involved in the in situ localization of vitronectin to reactive connective tissue (15-20). In this location, vitronectin is thought to be derived from serum.

In this study we examined the adhesive interactions within GBM tumors and found the functional expression of vitronectin and the vitronectin receptor integrin  $\alpha v\beta 3$  associated with the GBM tumor cell surface. Cultured GBM tumor cells specifically attached to cryostat sections of GBM tumors at sites containing vitronectin, and this adhesion event was inhibited by antibodies directed to vitronectin or the integrin  $\alpha v\beta 3$ . These data provide evidence for a cell adhesion mechanism in GBM tumors that may facilitate glioblastoma cell invasion of normal brain.

### Methods

**Reagents.** BSA, glucose, Tris base, NaCl, KCl, monovalent and divalent sodium phosphate, and methylene blue were purchased from Sigma Chemical Co. (St. Louis, MO). SDS and glycine were purchased from Bio-Rad Laboratories (Richmond, CA). Hepes and EDTA were purchased from Boehringer Mannheim (Indianapolis, IN). Divalent cations,  $MnCl_2$ ,  $MgCl_2$ , and  $CaCl_2$ , were purchased from Mallinckrodt, Inc. (Pairs, KY). All reagents were of the highest grade.

**Tissues.** Surgical biopsy material was frozen in liquid nitrogen and stored at  $-70^\circ C$ . The astroglial-derived tumors, all diffuse, were graded according to the four-tier Kernohan and Sayre grading system (21). All tumor biopsies were from adult ( $> 30$  yr) cerebral white matter before therapy, except for one residual radiated GBM tumor. Biopsies demonstrating reactive (nontumorous or benign astrocytic proliferation) astrogliosis were from patients ( $> 25$  yr) with encephalitis, seizures, or radiation changes adjacent to a radiated GBM tumor. In all of the tumors, the cryostat sections used for immunohistochemistry were representative of the most malignant area of the tumor biopsy.

**Antibodies.** Rabbit anti-human vitronectin IgG (anti-VN) and rabbit anti-human vitronectin receptor ( $\alpha v\beta 3$ ) IgG were produced by us or were purchased from Telios Pharmaceuticals (La Jolla, CA). Rabbit anti-human fibronectin receptor serum ( $\alpha 5\beta 1$ ) was purchased from Telios Pharmaceuticals. MAb anti-human fibronectin (F7387), MAb

anti-human fibrinogen, MAb anti-microtubule-associated protein (anti-MAP5), and rabbit anti-human glial fibrillary acidic protein were purchased from Sigma Chemical Co. Rabbit anti-human laminin and MAb anti-human collagen type IV were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). The following antibodies were gifts: MAb anti-human vitronectin (8E6) from Dr. D. Mosher (University of Wisconsin, Madison, WI), rabbit anti-human  $\beta 5$  integrin subunit from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA), MAb anti-human von Willebrand factor (anti-vWf) from Dr. Z. Ruggeri (Scripps Clinic), rabbit anti-human thrombospondin IgG from Dr. V. Dixit (University of Michigan, Ann Arbor, MI), MAb anti-human tenascin IgG (81C6) from Dr. M. Bourdon (La Jolla Cancer Research Foundation, La Jolla, CA), and MAb AP3 directed to human  $\beta 3$  integrin subunit from Dr. P. Newman (Southwestern Blood Center, Milwaukee, WI). The following antibodies were previously described, MAb anti-human  $\alpha v$  integrin subunit (LM142) (10), MAb anti-human  $\alpha v \beta 3$  integrin (LM609) (10), MAb anti-human  $\beta 1$  integrin subunit (LM534) (11, 12), and rabbit anti-human  $\alpha v \beta 3$  IgG integrin (10).

**Adhesive ligands.** Vitronectin was purified according to the method of Yatohgo et al. (22) and fibronectin was purchased from ICN Biomedicals. Synthetic peptides were purchased from Multiple Peptide System (San Diego, CA).

**Immunohistochemistry.** Immunohistochemistry (IHC) was performed on air-dried (5 min) cryostat sections, as the antivitronection and antivitronection receptor antibodies used showed a weak or negative reaction on formalin-fixed and paraffin-embedded tissue. IHC with anti- $\beta 5$  serum was performed on both air-dried and acetone-fixed (5 min, 4°C) cryostat sections. Sections of tumor, free of necrosis and hemorrhage, were used for IHC. IHC was performed as previously described (4), with the following modifications. Nonspecific protein-blocking and diluting buffer for the primary antibody was 5% BSA/PBS. Primary and horseradish peroxidase-labeled secondary antibody (1:250) (Bio-Rad Laboratories) were reacted each with the tissue for 1 h (room temperature). Substrate 3,3'-diaminobenzidine (1 mM) (Sigma Chemical Co.) was reacted with the tissue for 10 min. Tissues were counterstained in 1% methylene blue. Rabbit serum was used at 1:800, mouse MAb ascites were used between 1:500 and 1:2,000, mouse MAb spent culture media was used at 1:20, and IgG-purified rabbit and mouse antibodies were used at 10  $\mu$ g/ml. Staining was graded as: negative (0), weak (1+), moderate (2+), and strong (3+ and 4+). All negative control antibodies gave a completely negative reaction (0) and all positive control antibodies gave a moderate to strong reaction (2–4+). All antibodies that failed to react with tissue were graded as negative (0).

**Cell lines.** The U251 glioblastoma multiforme cell line was obtained from the American Type Culture Collection and was grown as previously described (3). Chromosome analysis of this cell line (University of California San Diego Chromosome Laboratory) demonstrated the same markers as previously described (3). The cell line was free of mycoplasma bacterial contamination throughout the course of these studies.

**Immunoprecipitation.** U251 cells in culture were surface-labeled with  $^{125}$ I, lysed in detergent, and the lysate was subjected to immunoprecipitation analysis as previously described (10–12). Immunoprecipitated proteins were analyzed by electrophoresis on nonreduced 7.5% SDS-PAGE, the gels were then stained, dried, and exposed to x-ray film (Eastman Kodak Co., Rochester, NY), as previously described (10–12). Nonradiolabeled high molecular weight standards (14,000–200,000) were purchased from Sigma Chemical Co. and electrophoresed with the samples.

**Tumor cell adhesion to cryostat sections of GBM tumors.** U251 cells were harvested with versene (0.53 mM EDTA, 21 mM Hepes, and 150 mM NaCl in PBS, pH 7.4) and washed in buffer consisting of 10 mM Hepes, 140 mM NaCl, 5.4 mM KCl, 5.56 mM glucose, 1% BSA, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 100  $\mu$ M MnCl<sub>2</sub>, pH 7.4 (adhesion buffer). Cells were resuspended at  $25 \times 10^4$ /ml. Before adhesion cryostat GBM sections on  $25 \times 40$ -mm glass slides were fixed with 3% buffered paraformaldehyde (a gentle fixative compared to formaldehyde and paraffin

embedding), washed in 0.1 M glycine/PBS followed by PBS, and the entire slide was blocked with 5% BSA/PBS (2 h, room temperature). Subsequently, the slide containing a 70-mm<sup>2</sup> cryostat serial GBM tumor section was flooded with 50,000 cells (U251) in 0.2 ml adhesion buffer (10 min, room temperature). The slide was flooded with the tumor cell suspension to ensure the tissue section was uniformly covered with cells. Sections of GBM tumor free of necrosis and hemorrhage, were used in this assay. After attachment, the slides were washed 3 times in agitated PBS (1 min/wash), postfixed in 3% paraformaldehyde, washed with 0.1 M glycine/PBS followed by PBS, dehydrated with 100% ethanol, and coverslipped. Alternatively, some sections were stained in 1% methylene blue before dehydration and coverslipping. Adhesion was allowed to take place in the presence of antibodies or controls as indicated in the figure legends. The time course of adhesion (10 min) was specifically chosen to examine the initial events of U251 cell attachment to GBM tissue. Longer incubation, 15–60 min, produced significant cell spreading on these cryostat sections. The 10-min incubation was chosen because the multiple molecular events involved in cell spreading on intact tissues for longer times would be difficult to interpret. After adhesion, washing, and fixation, the number of cells attached on each section were counted blindly using an inverted microscope with phase optics. In a typical experiment, in the absence of inhibitor (antibody or peptide), 300–350 cells were counted per (70-mm<sup>2</sup>) section. The cells attached to regions of the tumor expressing vitronectin. Based on the number of cells (50,000) per total slide area (1,000 mm<sup>2</sup>), and the 70-mm<sup>2</sup> area of the tumor section, ~ 10% of the cells available to the tissue section attached. This is likely a low estimate, assuming vitronectin is the predominant ligand the U251 cells are adhering to in the tumor section, and 30% of the tumor section expressed vitronectin.

## Results

To determine what matrix proteins were expressed on GBM tumors, serial cryostat sections were allowed to react with antibodies directed to vitronectin, fibrinogen, thrombospondin, fibronectin, von Willebrand factor, tenascin, laminin, and collagen type IV. Reactivity of these antibodies was detected by indirect immunoperoxidase staining, as described in Methods. As shown in Table I and Fig. 1 (A and C), among these proteins only vitronectin could be detected in association with the GBM tumor cells. Within the tumor mass, 25–40% of the tumor cells demonstrated vitronectin staining. The specificity of this reaction was demonstrated as the nontumorous adjacent white matter failed to react with anti-VN (Fig. 1 A, above arrowheads). Moreover, cryostat sections of normal adult cortex and cerebral white matter approximately age-matched (same decade) to the GBM patients failed to react with anti-VN (5/5) (Table I and Fig. 2 D). Adult glial-derived tumors arise predominantly in the cerebral white matter (21). To confirm the vitronectin reactivity, the anti-VN antibody was preincubated with purified soluble vitronectin or fibronectin and then reacted with serial GBM cryostat sections. As shown in Fig. 1 (B and D), the staining with anti-VN was completely blocked by purified vitronectin; however, fibronectin failed to effect anti-VN staining (Fig. 1, A and C). As previously described in untreated GBM tumors, fibronectin (4–6), laminin (6, 7), collagen types I, III, IV, V, and VI (5, 6), and von Willebrand factor (8) were specifically localized to the endothelial cell basement membrane and or pial/glial basement membrane, and were not found to be tumor cell associated. In this study, tenascin was restricted to the endothelial and pial/glial basement membranes; however, other studies (23) have found tenascin to be tumor cell associated in some GBM tumors. Taken together

Table I. Adhesive Ligands in Glioblastoma and Normal Adult Brain

	VN	FG	TSP	vWF	FN	TN	LM	COLIV	MAP5	GFAP
Glioblastoma										
Tumor cells	+*	-	-	-	-	-*	-	-	-	+ <sup>§</sup>
Normal cerebrum										
Glia	-	-	-	-	-	-	-	-	-	+ <sup>  </sup>
Neurons	-	-	-	-	-	-	-	-	+	-
Neuropil	-	-	-	-	-	-	-	-	+	-

Cryostat sections of normal adult cortex and cerebral white matter were reacted with antibodies to the following antigens by the immunoperoxidase technique, as described in Methods. Abbreviations for antigens are as follows: VN, vitronectin; FG, fibrinogen; TSP, thrombospondin; vWF, von Willebrand factor; FN, fibronectin; TN, tenascin; LM, laminin; COL-IV, collagen type IV; MAP-5, microtubule associated protein; GFAP, glial fibrillary acidic protein. \* Intense cell membrane and cytoplasmic reactivity restricted to discrete areas of tumor. † Tumor cell associated expression reported (5). § Reactivity of 10% of tumor cells. || Scattered astrocyte in molecular layer.

these results indicate that among a wide variety of potential matrix components, vitronectin appears to be a major adhesive ligand in GBM tumor parenchyma (5/5).

Vitronectin staining of GBM tissue was associated with the tumor cell membrane and/or cytoplasm (Fig. 2 A, arrows), while the adjacent vessels were predominantly nonreactive, as detected by the anti-VN MAb 8E6 (Fig. 2 A, arrowheads). This suggested that the GBM tumor cell membrane vitronectin was not derived from capillary leakage. In contrast, fibronectin staining was restricted to the blood vessels as observed in a serial section of this tumor (Fig. 2 B), or those found in the normal brain (Fig. 2 E). These results suggest that the vitronectin present in these tissues is tumor cell derived.

We examined earlier-stage astroglial tumors to determine whether vitronectin expression was related to progression of disease. To this end, vitronectin was only detected in malignant astroglial-derived tumors, i.e., GBM tumors (grade IV) (5/5 positive) and anaplastic astrocytomas (grade III) (2/5 positive), while lower grade astrocytomas failed to react with anti-VN (grades I and II) (0/4). Moreover, we could not detect vitronectin associated with glial or neuronal cells in normal adult cortex or cerebral white matter (0/5) (Fig. 2 D), or in reactive (nontumorous or benign astrocytic proliferation) astrogliosis (0/4). In addition, vitronectin was not detected in an adult cerebral white matter well-differentiated oligodendroglioma (0/1), a glial-derived tumor with a longer survival (21). This tumor was not included in the total numbers (Table II), since all other gliomas demonstrated clear predominant astrocytic differentiation. Vitronectin was focally detected in the endothelial cell extracellular matrix or basement membrane of low grade astrocytomas, as well as higher grade astroglial tumors, and in reactive astrogliosis, consistent with alteration of the blood brain barrier leading to leakage from serum. These results suggest that vitronectin may be a marker of the malignant astroglial cell and its expression correlates with tumor progression. In contrast, both the tumor, normal brain, and reactive astroglial cells reacted with anti-GFAP, a specific marker of glial intermediate filaments (4). In addition, normal brain neuropil or neuronal cell processes reacted with anti-MAP5, while normal rabbit serum (NRS) or NRS IgG failed to react with the tumors or normal adult cortex and cerebral white matter.

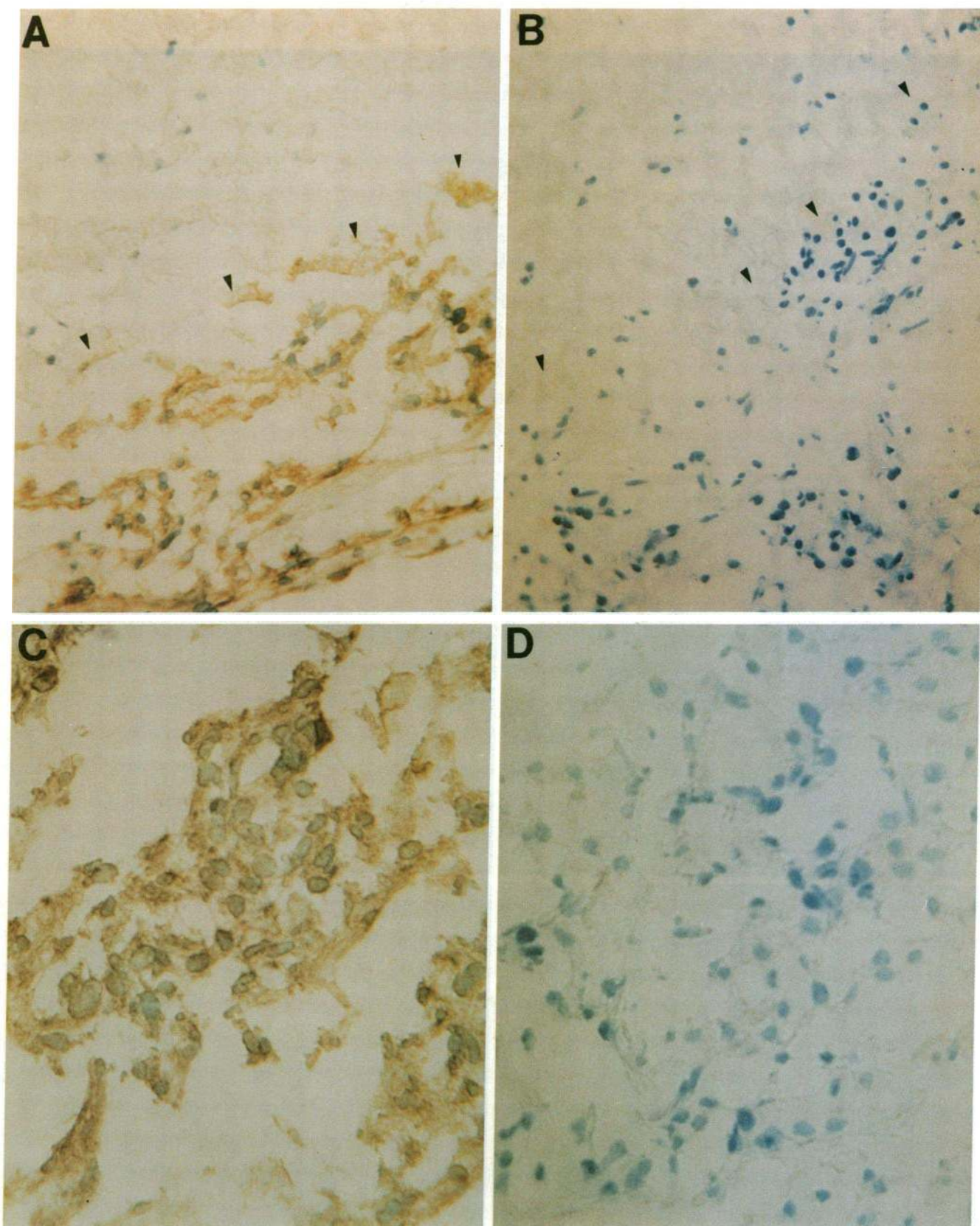
To determine whether vitronectin could be localized to other tumor cells in situ, we examined a variety of other tumors

for expression of this adhesive protein. Vitronectin was specifically detected as tumor cell associated in metastatic melanoma (2/2), a primary leptomeningeal melanoma, and a seminoma with embryonal cell carcinoma. However, we were unable to detect vitronectin in association with tumor cells in renal cell carcinoma or late-stage (invasive) breast, lung, and colon adenocarcinomas. It was present, however, in the perivascular connective tissue in these tumors, as previously described for breast and colon adenocarcinomas (24). Taken together, these data indicate that vitronectin expression is associated with malignant cells of neuroectodermal (glioblastoma and melanoma) and germ cell line derivation.

To assess whether GBM tumors express a receptor capable of recognizing vitronectin within the tumor parenchyma, we investigated whether these tumors expressed one or both of the two known nonplatelet vitronectin receptors (VNR), integrin  $\alpha v \beta 3$  or  $\alpha v \beta 5$  (9-12). Serial GBM cryostat sections were allowed to react with antibodies directed to integrin  $\alpha v$ ,  $\beta 3$ , and  $\beta 5$  subunits. As shown in Fig. 2 C, a MAb directed against  $\alpha v$  (10) reacted throughout the tumor, primarily with the tumor cell membrane. Similar but focal staining was observed with MAb AP3, directed to the integrin  $\beta 3$  subunit (25), consistent with the presence of the  $\alpha v \beta 3$  VNR in these tumors. In four GBM tumors examined with serial sections, the MAb AP3 and antivitronection staining colocalized. In contrast, a polyclonal antibody directed to the  $\beta 5$  subunit (26) failed to react with the GBM cryostat sections, suggesting that  $\alpha v \beta 3$  may be the predominant vitronectin-binding integrin in this tumor tissue. The integrin  $\alpha v \beta 3$  was detected on ~ 30% of glioma cells in anaplastic astrocytoma (grade III) (4/5), but it was not detected on low grade astrocytomas (grades I and II) (0/4). Glia and neurons in normal adult cortex and cerebral white matter (5/5), and glial cells in reactive astrogliosis (0/4) also failed to express  $\alpha v \beta 3$  (Table II). Therefore,  $\alpha v \beta 3$ , like vitronectin, may be a marker of malignant astroglial tumors. Endothelial cells in the GBM tumor (Fig. 2 C) and in normal white matter (Fig. 2 F) expressed the integrin  $\alpha v \beta 3$ , consistent with the identification of this receptor on endothelial cells in culture (27).

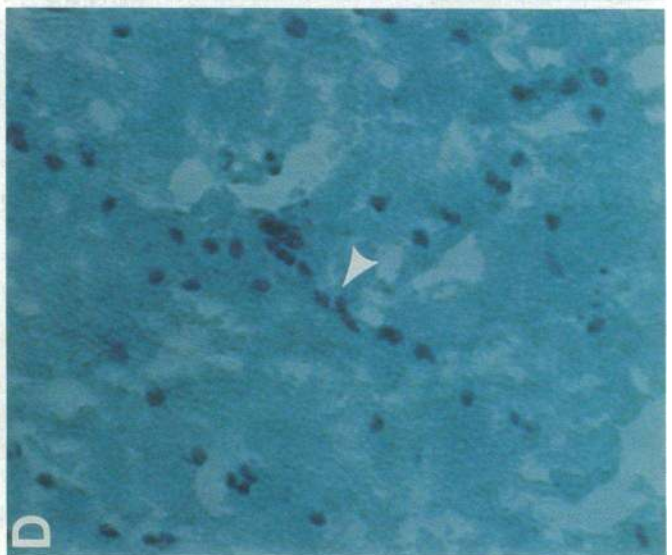
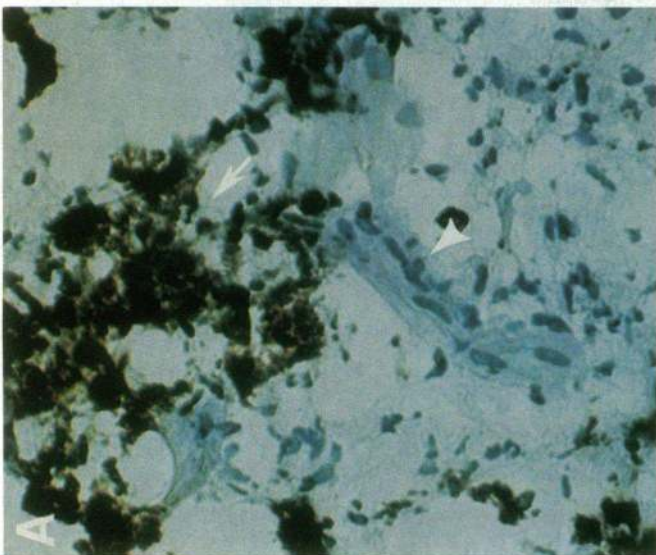
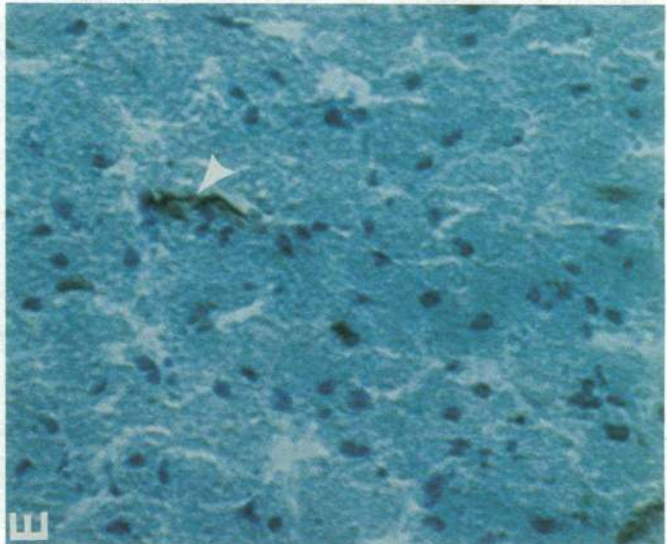
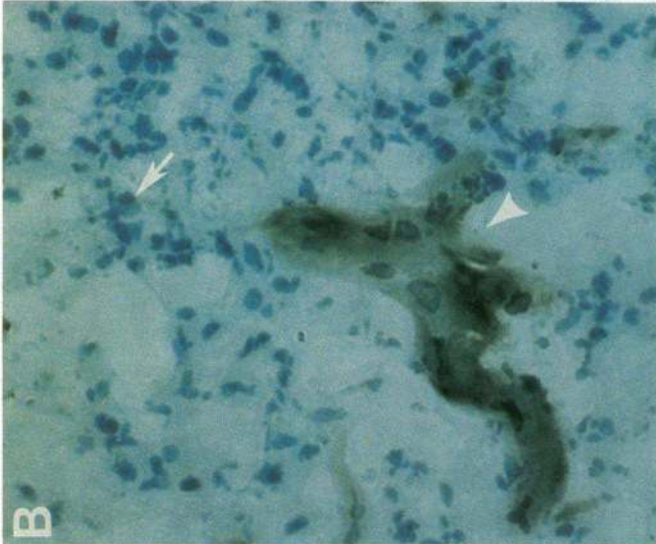
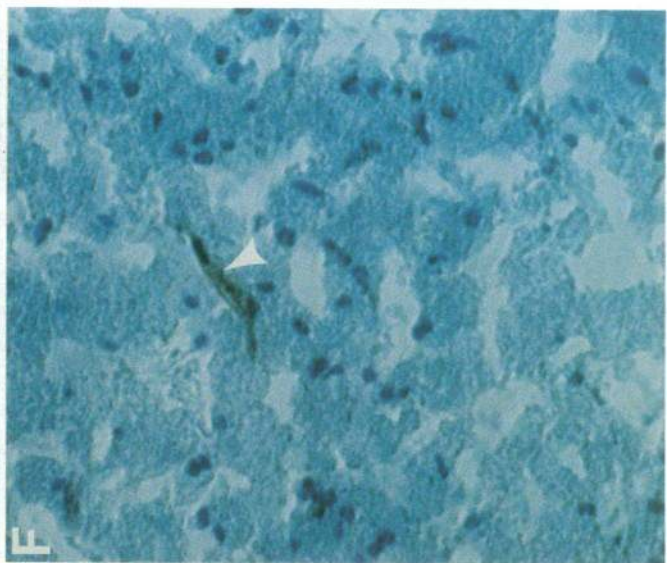
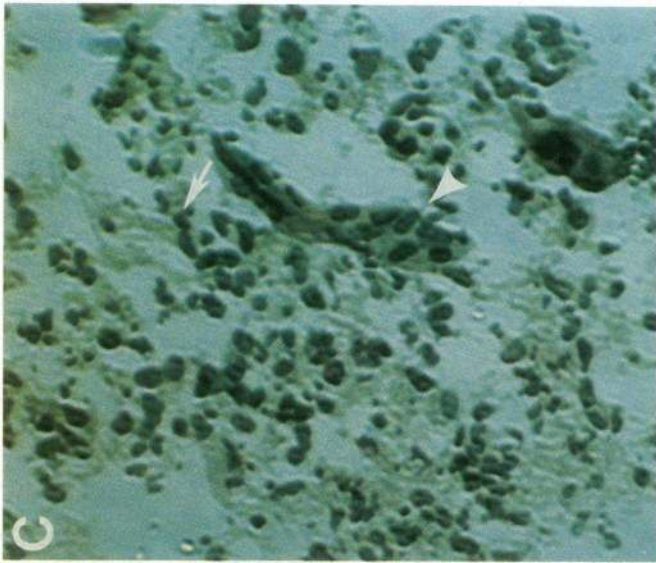
To determine whether cultured glioblastoma cells express the integrin  $\alpha v \beta 3$ , U251 glioblastoma cells (3) were surface labeled with <sup>125</sup>I and subjected to immunoprecipitation (IP) with antibodies directed to  $\alpha v \beta 3$ ,  $\alpha v$ ,  $\beta 3$ , and  $\beta 1$  integrin subunits, as previously described (10-12). As shown in Fig. 3, two





**Figure 1.** Vitronectin is expressed in glioblastoma tumor parenchyma. Human GBM tumor biopsy material (6- $\mu$ m cryostat serial sections) was stained by the immunoperoxidase technique as described in Methods. The primary antibody used in *A-D* was rabbit anti-VN IgG (10  $\mu$ g/ml). Before staining, anti-VN was allowed to react with purified fibronectin (*A* and *C*) or vitronectin (*B* and *D*), at final concentrations of 100  $\mu$ g/ml, 1 h. Tumor cell staining was graded as 2+ positive (scale from 1-4+) (*A* and *C*) and negative (0) (*B* and *D*). In *A* and *B* arrowheads depict the borders between tumorous (below) and nontumorous white matter (above). Photomicrographs were taken with a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY). (*A* and *B*) Magnification, 250; (*C* and *D*) magnification, 400.





**Table II. Histologic Expression of Vitronectin and the  $\alpha_v\beta_3$  Integrin on Malignant Glial Cells**

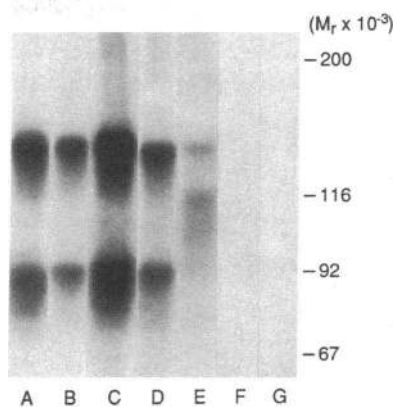
	VITRONECTIN	$\alpha_v\beta_3$
Normal adult* cerebral glia	0/5	0/5
Reactive astrogliosis	0/4	0/4
Grade I–II astrocytoma	0/4	0/4
Grade III astrocytoma (anaplastic)	2/5 <sup>†</sup>	4/5
Grade IV astrocytoma GBM	5/5	5/5

On all tissues expressing vitronectin, 25–40% of tumor cells were positive. Immunohistochemistry was performed on cryostat sections as described in Methods. \* Normal adult cortex and cerebral white matter from individuals approximately age-matched to the GBM patients. <sup>†</sup> Two positives per five tumors examined.

bands of  $M_r$  150 and 95 were detected with a polyclonal anti- $\alpha_v\beta_3$  antibody, and MAbs directed to  $\alpha_v\beta_3$ ,  $\alpha_v$ , and  $\beta_3$ , lanes A, B, C, and D, respectively. This is consistent with the  $M_r$  of the  $\alpha_v\beta_3$  integrin previously described on melanoma and endothelial cells (10, 27). Therefore, glioblastoma cells in situ and in culture express  $\alpha_v\beta_3$ . Consistent with a previous study (28), these cells express  $\beta_1$  integrins (Fig. 3, lane E).

To determine whether vitronectin might serve an adhesive role in glioblastoma tumors in situ, we examined the ability of U251 GBM cells to attach to cryostat sections of GBM tumor tissue. Cryostat sections were chosen to colocalize the GBM vitronectin expression with the U251 cell adhesion, as anti-vitronectin antibodies react weakly or fail to react with formalin-fixed tissue. U251 glioblastoma cells readily attached to GBM cryostat sections, and 95% of this attachment was in regions expressing vitronectin. This U251 cell attachment to GBM cryostat sections was quantitated in a GBM tumor where 30% of the tumor cells expressed vitronectin, as determined by serial section immunoreactivity with the anti-VN MAb 8E6 (Fig. 4, A and B). Of the attached U251 cells, 95% localized to the glioblastoma tumor parenchyma where vitronectin was expressed and 5% localized to the blood vessels. In a typical adhesion experiment 300–350 U251 cells attached/70 mm<sup>2</sup> cryostat section and, as stated in Methods, ~10% of available cells attached to the cryostat section. U251 cells failed to attach to normal adult neocortex or cerebral white matter. These results suggest that vitronectin expressed in GBM tumors may serve as a relevant adhesive ligand.

To test this hypothesis, U251 cells were allowed to attach to GBM cryostat sections that were preincubated with anti-VN. As shown in Fig. 5, rabbit anti-VN sera blocked this adhesion by ~50%. These results suggest vitronectin is among the relevant adhesive ligands present in GBM tumors in situ. In addition, U251 cell adhesion to these tissues could be inhibited by 60% with a soluble RGD-containing peptide (GRGDSP) as



**Figure 3.** Cultured GBM cells express the  $\alpha_v\beta_3$  integrin. U251 cells were surface labeled with <sup>125</sup>I, lysed, subjected to immunoprecipitation (IP) with antibodies directed to the  $\alpha_v\beta_3$  integrin and, analyzed by SDS-PAGE under nonreducing conditions on 7.5% gels, which were subjected to autoradiography as described in Methods.

Two bands were detected at  $M_r$  150 and 95 with anti-VNR IgG (anti- $\alpha_v\beta_3$ ) (lane A), MAb anti- $\alpha_v\beta_3$  (LM609) (lane B), MAb anti- $\alpha_v$  (LM142) (lane C) and MAb anti- $\beta_3$  (AP3) (lane D), consistent with the  $M_r$  of the  $\alpha_v$  and  $\beta_3$  chains, respectively, previously described on melanoma cells (10). Controls consisted of MAb anti- $\beta_1$  (LM534) (lane E), NRS IgG (lane F), and anti-mouse Sepharose (lane G).

compared to a random peptide (GDRGSP) (Fig. 5), consistent with the possibility of this being an RGD-dependent integrin-mediated adhesion event.

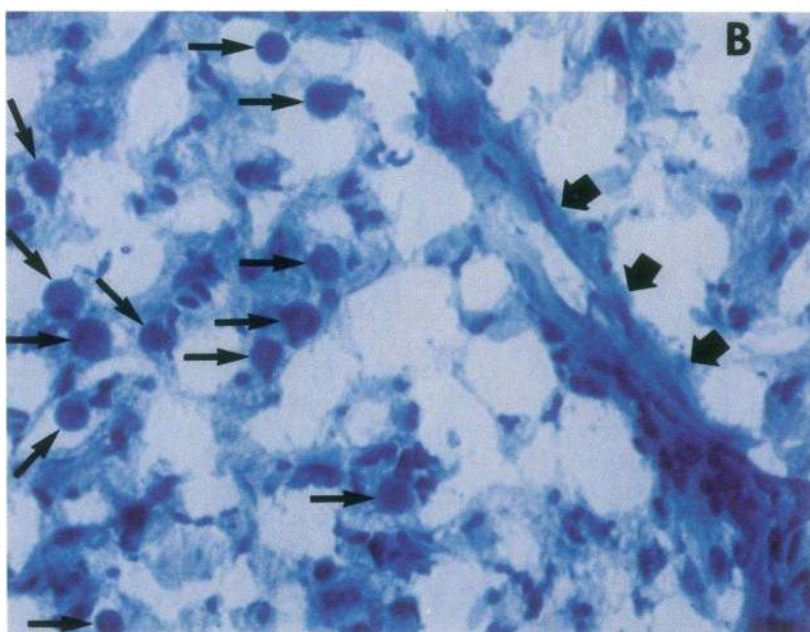
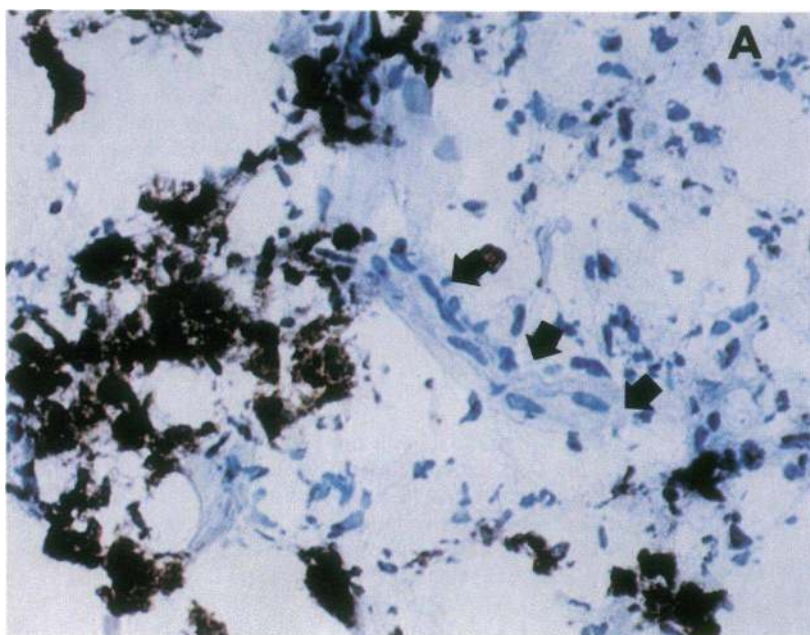
To determine whether integrin  $\alpha_v\beta_3$  expressed on U251 cells was involved in this adhesion event, U251 cells were preincubated with rabbit anti- $\alpha_v\beta_3$  IgG or MAb anti- $\alpha_v\beta_3$  (LM609), and then allowed to attach to the GBM cryostat sections. As shown in Fig. 5, these antibodies inhibited U251 cell attachment to GBM cryostat sections by ~70%. A similar inhibition (70–90%) was observed with these antibodies when U251 cells were allowed to attach to microtiter wells coated with purified vitronectin (not shown). In contrast, rabbit anti- $\alpha_5\beta_1$ , NRS, and NRS IgG failed to inhibit U251 cell adhesion to GBM tumor tissue (Fig. 5), while anti- $\alpha_5\beta_1$  blocked attachment of these cells to purified fibronectin (not shown). These results provide evidence that the vitronectin expressed in GBM tumors may serve an adhesive role in the malignant phenotype of astroglial-derived cells, and that integrin  $\alpha_v\beta_3$  expressed on the tumor cell surface potentiates this interaction.

## Discussion

Adhesive interactions are involved in a diverse array of biological processes, including embryogenesis, thrombosis, wound healing, and tumor cell invasion (1, 2). Many of these adhesive events are mediated by members of the integrin family of cell adhesion receptors that recognize ligands present in the extracellular matrix or on opposing cells (1, 2). In fact, tumor cells typically synthesize their own matrix components (29), and show altered integrin expression (1, 2, 30, 31) as compared to

**Figure 2.** Vitronectin expression in a GBM tumor is restricted to the tumor parenchyma. GBM tumor tissue from a second patient (A–C, 6- $\mu$ m cryostat sections) or normal adult cerebral white matter from surgical biopsy (D–F, 9- $\mu$ m cryostat sections) were stained as in Fig. 1. Primary antibodies were anti-human VN MAb 8E6 (1:20 spent culture fluid) (A and D); MAb anti-human fibronectin (1:500 ascites) (B and E); or anti- $\alpha_v$  MAb LM142 (10) (1:1000 ascites) (C and F). Arrowheads depict blood vessels and arrows depict glioblastoma tumor cells. Staining was graded as 4+ positive in A, 3+ positive in B, 2+ positive (tumor cells) and 3+ positive (endothelial cells) in C, negative (0) in D, 3+ positive (endothelial cells) in E, and 2+ positive (endothelial cells) in F. (A–F) Magnification, 100.





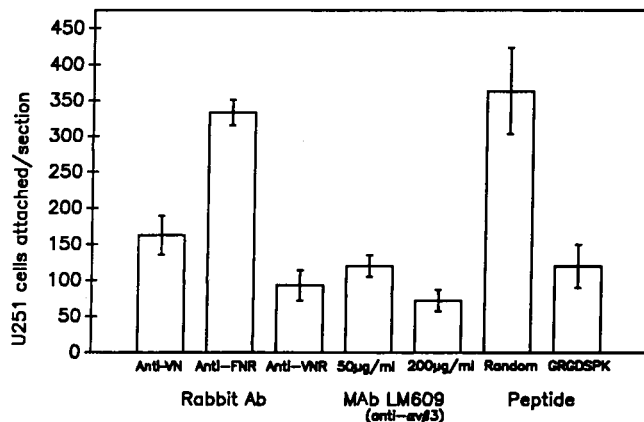
**Figure 4.** U251 cultured glioblastoma cells adhere to cryostat sections of a GBM tumor. In *A*, a GBM tumor section was stained with anti-VN, MAb 8E6, as in Fig. 2. Short arrows depict a blood vessel. In *B*, U251 cells were allowed to attach to a serial cryostat section, as described in Methods. Short arrows depict a blood vessel and long arrows depict adherent U251 glioblastoma cells. The tumor nuclei in the GBM tumor section (*A* and *B*) are irregular in shape and blue. (*B*) is a representative field accounting for ~ 1% (0.5 mm<sup>2</sup>) of a 70-mm<sup>2</sup> cryostat section. (*A* and *B*) Magnification, 100.

their normal cell counterparts. These observations suggest that tumor cells can remodel their surrounding extracellular matrix to facilitate their invasive phenotype.

In this report, we demonstrate that the adhesive protein vitronectin is found within the parenchyma of late-stage astroglial-derived tumor tissue. Vitronectin may be one of the primary adhesive ligands in these tissues due to the fact that we were unable to detect, as tumor cell associated, a wide variety of other extracellular matrix components including, collagen, fibronectin, fibrinogen, thrombospondin, von Willebrand factor, tenascin, and laminin. In addition, the expression of vitronectin in these tumors may be related to the progression of disease because it could not be detected on low grade astroglial-derived tumors, reactive astrogliosis, or on glial cells in the normal adult brain. The vitronectin detected in GBM tumors

localizes to the tumor cell cytoplasm and plasma membrane. Thus, unlike fibronectin and other plasma proteins, its expression is independent of blood vessels. These results suggest that the vitronectin present in these tissues is derived from the tumor cells. However, long term cultured cell lines failed to synthesize detectable levels of vitronectin suggesting that, upon culture, cells may lose this property. This may not be surprising because most GBM cell lines (80%) also fail to express GFAP, an intermediate filament and a characteristic marker of glial-derived cells and their tumors *in situ* (3, 4).

The expression of vitronectin is not restricted to GBM tumors because it was also shown to be tumor cell associated in melanoma and embryonal cell carcinoma. However, vitronectin was not expressed in carcinoma of the breast, colon, lung, and kidney. It is important to point out that, consistent with



**Figure 5.** Effect of antivitronection, antiintegrin antibodies, or an RGD peptide on U251 cell adhesion to GBM cryostat sections. U251 cells were allowed to adhere to 70-mm<sup>2</sup> GBM cryostat sections as in Fig. 4 B. However, adhesion was allowed to take place in the presence of rabbit anti-VN (1:50), rabbit anti-FNR (anti- $\alpha 5 \beta 1$ ) (1:50), anti-VNR IgG (anti- $\alpha v \beta 3$ ) (250  $\mu g/ml$ ), MAb anti- $\alpha v \beta 3$  (LM609) at 50 or 200  $\mu g/ml$ , random peptide GDRGSP (200  $\mu g/ml$ ) and GRGDSP (200  $\mu g/ml$ ). Results are expressed as the actual number of adherent cells. NRS (1:50) and NRS IgG (250  $\mu g/ml$ ) had no effect on adhesion when preincubated with the GBM cryostat sections or with the U251 cells. In a typical experiment 300–350 cells attached in the absence of inhibitor. Based on the 70-mm<sup>2</sup> tissue section, ~ 10% of the total U251 cells available to the tissue section attached. Cell attachment was primarily restricted to 30% of the cryostat section, localizing to regions of vitronectin expression as in Fig. 4. Each bar represents the mean  $\pm$  the standard error of 3–4 replicates.

previous reports (24), vitronectin, as well as other adhesive ligands, were localized to the perivascular connective tissue in these tumors. These results suggest that vitronectin may be a marker of neuroectodermally-derived tumors.

We examined whether the vitronectin expressed within GBM tumors might serve as a relevant adhesive ligand in this tissue. To this end, cultured GBM tumor cells were examined for their ability to specifically attach to cryostat sections of GBM tumor tissue. This adhesion was primarily restricted to regions of the tissue that expressed vitronectin. In addition, pretreatment of this tissue with an antibody to vitronectin significantly inhibited the attachment of GBM cells to this tissue, suggesting that vitronectin is among the relevant adhesive ligands in these tumors. In support of this contention, the adhesion of GBM cells to this tissue was also inhibited with an RGD-containing peptide and a monoclonal antibody to the vitronectin-binding integrin  $\alpha v \beta 3$ . That integrin  $\alpha v \beta 3$  was detectable in GBM tumor tissue and colocalized with vitronectin in four GBM tumors suggests it may promote adhesion of GBM tumor cells to vitronectin in situ. It is of interest to point out that the normal brain adjacent to these tumors is essentially devoid of vitronectin. As previously described, and we verified, fibronectin (4–6), collagen (5, 6), laminin (6, 7), and von Willebrand factor (8) were restricted to the endothelial and pial/glia basement membranes in normal brain. Therefore, it is conceivable that the vitronectin present in GBM tumors and integrin  $\alpha v \beta 3$  may play a role in the ability of these cells to invade normal brain. At present we cannot rule out the presence of other vitronectin receptors in these tumors. However, we were not able to identify the  $\beta 5$  subunit in these tumor sections,

which suggests that the VNR integrin  $\alpha v \beta 5$  is probably not playing a significant role in this adhesive interaction.

It is also conceivable that other adhesive mechanisms are involved in this adhesion event. For example, N-CAM and/or cadherin-type homotypic cell–cell interactions might result in the adherence of U251 cells to tumor cells within the cryostat sections. The experimental condition chosen for this assay (10 min incubation) was designed to measure the initial events of adhesive interactions occurring between the tumor cell surface and GBM tumor tissue. More importantly, by designing the experiment in this way we minimized the tendency of GBM cells to spread, which would significantly enhance the strength and complexity of this adhesive event. Cell spreading on intact tissues would be difficult to interpret because of multiple molecular events that occur with time.

In addition to serving as an adhesive ligand in GBM tumors, vitronectin may be involved in other biological mechanisms that might contribute to the malignant behavior of these tumors. For example, vitronectin helps to regulate fibrinolytic mechanisms through an interaction with the type 1 plasminogen activator inhibitor (32, 33). Vitronectin is also known to bind the C5-9 membrane attack complex of complement, and thus may prevent complement-mediated cytolysis by host immune cells (19, 20). Recently, vitronectin was shown to interact with  $\beta$ -endorphin (34), and the latter was independently demonstrated to bind to the surface of glioblastoma cells (35), suggesting that vitronectin associated with these tumors may serve to enhance the binding of  $\beta$ -endorphin.

In summary, this report provides the first evidence that vitronectin is specifically localized to tumor cells in situ. Its expression appears to correlate with the late stages or the most malignant astroglial-derived tumors. Although vitronectin has a number of biological properties, it is capable of serving an adhesive role when expressed in the parenchyma of GBM tumors. The conspicuous absence of other adhesive ligands in normal brain tissue, excluding blood vessels, suggests that the vitronectin present in GBM tumors may facilitate their malignant behavior.

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