Intracranial Microenvironment Reveals Independent Opposing Functions of Host \( \alpha V \beta 3 \) Expression on Glioma Growth and Angiogenesis*\({\dagger}\)

Received for publication, June 5, 2006, and in revised form, September 29, 2006 Published, JBC Papers in Press, October 6, 2006 DOI 10.1074/jbc.M605344200

Masayuki Kanamori, Tomohiro Kawaguchi, Mitchel S. Berger, and Russell O. Pieper

From the Department of Neurological Surgery and the Brain Tumor Research Center, University of California, San Francisco, California 94115-0875

\( \alpha V \beta 3 \) integrins are overexpressed in the host-derived vasculature of glioblastoma multiform (GBM) and are believed to contribute to angiogenesis and tumor growth. To directly address the role of host \( \alpha V \beta 3 \) expression in GBM growth and behavior, we intracranially implanted integrin \( \beta 3 \)-expressing GBM cells into \( \beta 3 \) wild type (WT) or \( \beta 3 \) knock out (KO) mice and monitored angiogenesis and growth. GBM in \( \beta 3 \) WT animals had a vessel density greater than that in \( \beta 3 \) KO animals, consistent with a pro-angiogenic, pro-tumorigenic view of host integrin function. GBM in \( \beta 3 \) WT animals, however, were no larger than those in \( \beta 3 \) KO animals, because GBM in \( \beta 3 \) WT animals were infiltrated with a higher number of tumor necrosis factor \( \alpha \)-secreting, apoptosis-inducing macrophages than the tumors in the corresponding \( \beta 3 \) KO animals. The tumor-suppressive effects of host \( \beta 3 \) expression could be reversed by macrophage depletion or by transplantation of bone marrow from \( \beta 3 \) KO animals into \( \beta 3 \) WT animals, both of which significantly increased tumor growth independently of tumor vessel density. Taken together, these results show that host \( \alpha V \beta 3 \) integrin expression has opposing actions in the intracranial setting, enhancing tumor vascularization and growth while independently enhancing macrophage-mediated tumor elimination. Appropriate management of these functions could lead to enhanced efficacy of anti-integrin based therapies for glioma.

Brain tumors affect \(~100,000\) Americans per year and remain among the most difficult of human tumors to successfully treat. Individuals with glioblastoma multiform (GBM), a highly aggressive and angiogenic form of glioma, have a particularly poor prognosis, with an average survival of \(<2\) years (1–3). Although surgical resection followed by radiation and chemotherapy have been shown to slightly increase the lifespan of individuals with GBM, new approaches that can selectively target the tumor are needed.

The search for agents that can selectively target glioma cells has led to an interest in the role of integrins in glioma biology. Integrins are transmembrane glycoprotein complexes of non-covalently linked \( \alpha \) and \( \beta \) subunits. There are 8 known \( \beta \) subunits that combine with 18 \( \alpha \) subunits in a defined manner to create more than 24 unique \( \alpha \beta \) heterodimers. Although some \( \beta \) subunits combine with a large number of \( \alpha \) subunits, others exhibit greater selectivity and combine with single \( \alpha \) subunits (4). As an example, the \( \beta 3 \) subunit combines exclusively with the \( \alpha V \) subunit in most tissues except in platelets, where it also exhibits a pro-clotting interaction with the \( \alpha IIb \) integrin subunit (5). Integrin heterodimers each recognize a specific range of ligands in the extracellular matrix or on neighboring cells, and integrin binding plays a key role in adhesion of integrin-expressing cells to the extracellular matrix. Integrin-mediated cellular anchorage, however, is also accompanied by linkage of activated integrins to the actin cytoskeleton and the triggering of a large number of signal transduction pathways that can influence nearly every aspect of cellular behavior (6–8).

Because integrins play a key role in processes requiring cell-cell interaction such as cell migration and angiogenesis, they also appear to play a key role in tumorigenesis. \( \alpha V \beta 3 \) integrin complexes in particular are overexpressed in the proliferating vascular endothelial cells surrounding the tumor (9–11). This is especially true in GBM, in which \( \alpha V \beta 3 \) is overexpressed at the invasive, highly angiogenic edges of the tumor (12). These observations have led to the suggestion that \( \alpha V \beta 3 \) integrins may play a role in the angiogenesis and/or growth and invasion of GBM. This concept has been further supported by the demonstration that monoclonal antibodies and small cyclic arginine-glycine-aspartate peptide ligands of \( \alpha V \beta 3 \), both of which function presumably as \( \alpha V \beta 3 \) antagonists, led to tumor regression in animal models of GBM (13). These studies as a whole have defined \( \alpha V \beta 3 \) as a promising target for tumor-selective therapy of GBM.

More recent detailed studies, however, have questioned the established role of tumor and host \( \alpha V \beta 3 \) expression in glioma growth. We previously showed that overexpression of \( \alpha V \beta 3 \) in intracranially implanted GBM cells did not increase the blood vessel density or size of the subsequent tumors but, rather, suppressed both tumor oxygenation and growth (14). Because the growth suppressive effects of \( \beta 3 \) expression in the tumor could

---

*This work was supported by National Institutes of Health Grant CA 94989 (to R.P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\†The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: UCSF Cancer Center, 2340 Sutter St., Rm. N219, San Francisco, CA 94115-0875.

3 The abbreviations used are: GBM, glioblastoma multiform; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; KO, knock out; BMT, bone marrow (BM) transplant; FITC, fluorescein isothiocyanate; DIG, digoxigenin; PBS, phosphate-buffered saline; WT, wild type; HDMEC, human dermal microvascular endothelial cell line; VWF, von Willebrand factor; DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.
be overcome by genetic alterations (Akt activation, VEGF over-expression) common to GBM, however, these studies suggested that αVβ3 expression in the host, rather than in the tumor, may play a more important role in modulating glioma growth. The effects of host αVβ3 expression on tumor growth have been examined, and in these studies host αVβ3 expression surprisingly suppressed rather than enhanced the growth of subcutaneously implanted human tumor cells in association with suppression of angiogenesis (15, 16). These studies, however, were done in the relatively avascular subcutaneous setting and, therefore, might not adequately address the role of host αVβ3 integrin expression in the highly vascular intracranial setting that is home to GBM. Because angiogenesis and tumor growth are highly tissue-specific processes, both of which are greatly influenced by the tumor microenvironment (17, 18), we considered the possibility that effects of host αVβ3 expression might be tissue-dependent and far different in the intracranial versus the subcutaneous setting. We here report that tumor-related effects of host αVβ3 expression are indeed highly dependent on tumor microenvironment and that in the intracranial setting host αVβ3 expression increases glioma vessel density while independently suppressing glioma growth by enhancing TNFα-secreting macrophage infiltration into the tumor. Manipulation of these independent opposing functions of αVβ3 expression functions could lead to enhanced efficacy of anti-integrin-based therapies for glioma.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—U251, an established human GBM cell line, was obtained from the University of California, San Francisco (UCSF) Brain Tumor Research Center Tissue Bank. GL261, a mouse GBM cell line created by intracranial implantation of a methylcholanthrene pellet in a C57BL/6 animal (19), was provided by Dr. Y. Gillespie, University of Alabama at Birmingham. Cells were maintained as monolayers in a complete medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. An immortalized human dermal microvascular endothelial cell line (HDMEC) was provided by Dr. Gabriele Bergers (UCSF Dept. of Neurological Surgery). HDMEC cells were cultured in MCDB131 media (Invitrogen) with 10% fetal bovine serum and 1:100 L-glutamine. All cells were cultured at 37 °C in a humidified atmosphere consisting of 5% CO₂. All reagents for cell culture were obtained from UCSF Cell Culture Facility.

**Mouse and Tumorigenesis Assay**—β3 integrin knock out (KO) (15) mice were intercrossed with immunodeficient mice lacking the Rag1 gene to generate immunodeficient Rag1 KO mice with or without β3 integrin expression (C57BL6/129Sv). The genotypes were confirmed by the polymerase chain reaction as described previously (20). Intracranial injections of U251 and GL261 cells into mice were performed as described previously (14, 21). Briefly, 3 × 10⁶ cells were stereotactically injected into striatum of age- and sex-matched anesthetized mice (14, 22). Mice were sacrificed 35 days after injection. Tumor-bearing brains were sectioned coronally at the point of cellular implantation. After hematoxylin and eosin staining, sections were photographed, and the length (a) and width (b) of the largest tumor cross-sectional areas were determined. To obtain another parameter of widths (c), samples were cut into 10-μm serial coronal sections, and every 10th section was stained for hematoxylin and eosin. Tumor volume was calculated using the standard formula \( V = \text{length} \times \text{width} \times \text{width} \times \text{height} \). For subcutaneous studies, 5 × 10⁶ cells were injected into the flank, and 25 days after injection the volume was calculated using the standard formula \( \text{length} \times \text{width} \times \text{height} \) (23–27).

**Mouse Bone Marrow Transplants (BMT)**—Six-week-old recipient female immunodeficient Rag1 KO mice with or without β3 integrin expression (C57BL6/129Sv) were lethally irradiated with 8 Gy (4 Gy × 2 at a 4-h interval) and reconstituted by tail vein injection of 5 × 10⁶ BM cells isolated from male mice generated from the same breeding pair of animals and with the same genetic background. These mice were treated with anti-biotic water for 14 days. Four weeks after BMT the mice were used for further analyses.

**Immunohistochemistry**—5-μm paraformaldehyde-fixed, paraffin-embedded sections or 6-μm frozen sections were used for immunohistochemistry. Primary antibodies used for immunohistochemistry were rat anti-F4/80 (Serotec, Oxford, UK), rabbit anti-TNFα (R&D Systems, Minneapolis, MN), rabbit anti-Von Willebrand factor (vWF) (Abcam, Cambridge, MA) and rat anti-endoglin (R&D). All were used at 1:200 dilution except anti-vWF antibody, which was used at 1:800 dilution. For immunofluorescence, FITC- or rhodamine-conjugated secondary antibodies were used at the dilution of 1:200. These slides were counterstained by DAPI and mounted with antifade solution (Prolong, Molecular Probes, Eugene, OR). Vessel density was assessed by counting the number of vWF- or endoglin-positive vessels in 10 independent tumor fields at 400 with the aid of an ocular grid (14). Vessel density was reported as the mean ± S.D.

**In Situ Hybridization Combined with Immunohistochemistry**—**In situ** hybridization was performed as described elsewhere (28). Briefly, digoxigenin (DIG)-labeled cDNA probe was prepared by using a PCR DIG Probe Synthesis Kit (Roche Diagnostics). Paraffin-embedded tumor sections were prepared by deparaffinization, rehydration, and digestion with proteinase K for 15 min at 37 °C. Hybridization was performed at 42 °C overnight in a hybridization mixture consisting of 50 μl of deionized formamide, 10 μl of salmon sperm DNA (Invitrogen), 10 μl of dextran sulfate, 10 μl of 50× Denhardt’s solution, 10 μl of 20× standard saline citrate, and 500 ng of denatured DIG-labeled probe. The DIG-labeled Y chromosome was visualized using anti-DIG fluorescein-conjugated antibody (Roche Diagnostics). To detect the Y chromosome-positive macrophages, slides were initially immunostained for F4/80 expression, after which in situ hybridization was performed.

**Flow Cytometry**—FITC-conjugated rat monoclonal antibodies (rat anti-mouse anti-F4/80, Serotec; anti-NK-cell, BD Biosciences) were used to stain monocytes/macrophages or NK cells present in the blood of mice, respectively. Blood samples were incubated with 1:100 dilution of antibody for 1 h at room temperature, treated with red cell lysis buffer (BD Pharmingen) (29), washed twice with phosphate-buffered saline (PBS), resuspended in PBS, and analyzed in a FACScan flow cytometer with CELLQUEST software (BD Biosciences). Aliquots of macro-
Effects of Host αVβ3 Integrin Expression on Gliomas

Phages from β3 WT and KO mice were sorted and plated onto culture dishes for further analysis.

**Macrophage Conditional Supernatant/Western Blot Analysis**—Macrophages isolated from β3 WT or KO mice were activated by incubation with 100 ng/ml lipopolysaccharide (Chemicon, Temecula, CA). The culture was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C for 24 h. At the end of the culture, supernatants were collected, separated into aliquots, and stored at −80 °C for further analysis. The supernatant from the macrophage culture without lipopolysaccharide stimulation was used as a control. Activated macrophages were harvested and lysed as described previously (14). Whole cell lysate (10 μg) was subjected to gel electrophoresis and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% skim milk and incubated with antibodies against TNFα (1:1000, R&D Systems, Minneapolis, MN) or α-tubulin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody using ECL Western blotting detection reagents (Pierce).

**Analysis of Cell Cycle Distribution/Annexin V-FITC Assay**—Cells were harvested and washed in PBS and fixed in 70% ethanol at −20 °C. The cells were washed once with PBS followed by incubation in PBS containing 40 μg/ml propidium iodine (Sigma) and 200 μg/ml RNase A (Sigma) for 1 h at room temperature in the dark. Stained nuclei were then analyzed on FACSscan machine (BD Biosciences) with 10,000 events/determination. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to assess cell cycle distribution. The annexin V-FITC binding assay was done using an ApoAlert Annexin V kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Briefly, after incubation with macrophage supernatant, U251 cells were washed with PBS and trypsinized. Cells were rinsed and then suspended in binding buffer. After incubation with annexin V-FITC, propidium iodine was added to the cell suspension. Annexin V-FITC positive and propidium iodine-negative cells were counted on FACSscan machine (BD Biosciences) with 20,000 events/determination.

**Apoptosis Analysis**—For cell death analysis, DNA fragmentation (terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) staining) was performed by using the FD Apop kit (FD Neurotechnologies, Ellicott City, MD) according to the manufacturer’s instructions. Nuclei were counterstained with methyl green.

**In Vivo Macrophage Depletion**—Macrophages were depleted by intraperitoneal injection of an anti-macrophage antibody (Accurate Chemical and Scientific, Westbury, NY) according to the manufacturer’s protocol. Antibody injection was begun 2 days before tumor implantation and was repeated at days 0 (same day as implantation), 2, 5, and 7 and twice a week thereafter until animal sacrifice. Preliminary tests using these antibodies confirmed that this protocol significantly reduced the number of circulating macrophages while not effecting levels of marrow-derived NK cells. As a control, rabbit normal IgG was used in the same strategy.

Transendothelial Migration Assay—To assess in vitro macrophage transmigration ability, we performed transendothelial migration assays (30). Briefly, transwell culture inserts were coated with 50 mg/ml laminin for 30 min. Excess laminin was removed from the inserts and immortalized HDMEC at 10^5/ml were seeded on the inserts in 100 μl of medium. Cells were allowed to grow to confluence on filters for 48 h. The macrophages isolated from β3 WT or β3 KO mice were adjusted to 10^5 cells/ml, and 100 μl of cell suspension were added per insert. Medium (300 μl) containing 125 ng/ml monoclonal chemoattractant protein-1 was placed into the lower chambers of the transwells, and the inserts were carefully placed into the lower chambers to avoid air bubbles forming at the interface between the underside of the insert and the medium. Migration was then allowed to proceed for 5 h at 37 °C, after which the number of cells that had migrated into the lower chamber was determined. For blocking studies, anti-integrin β3 antibody (Chemicon) was added to a final concentration of 50 mg/ml. The tubes were then incubated on a shaker for 1 h at 4 °C. Before the assay, cells were spun down, washed once in medium, and then resuspended in 300 μl of fresh medium. This was then added to three wells per condition. For each experiment, the number of cells that had transmigrated was expressed as the mean value of cells counted in three wells.

**Statistical Analysis**—All statistical analyses were performed using the Student’s t test, with significance defined as p < 0.05.

**RESULTS**

**Host Integrin β3 Expression Has Different Effects on Subcutaneous Versus Intracranial Tumors**—Previous studies have shown that host αVβ3 expression suppressed rather than enhanced the growth of human tumor cells in association with suppression of angiogenesis (15, 16), although all these studies were done in the relatively avascular subcutaneous setting. To determine how host β3 expression might contribute to tumor formation in a vessel-rich brain tumor microenvironment, we implanted human U251 GBM cells subcutaneously and intracranially into immunodeficient (Rag1 KO) β3 WT or β3 KO mice and monitored the tumor vessel density and size of the resultant tumors. Host β3 expression suppressed the subcutaneous growth of U251 cells (Fig. 1A) and also decreased the density of blood vessels in the tumor as detected by immunohistochemistry using antibodies recognizing the endothelial cell markers vWF (Fig. 1B) or endoglin (not shown). These results are consistent with previous reports showing host β3-mediated suppression of the growth and angiogenesis of other subcutaneously implanted human tumor cell lines (15, 16). In the intracranial setting, however, effects of host β3 expression on tumor growth and vascularity were quite different. U251 GBM cells intracranially implanted into β3 WT animals formed tumors that were no different in size from those that formed in β3 KO animals (Fig. 1C). Furthermore, an examination of vessel density showed that unlike what was seen in the subcutaneous setting, host β3 expression enhanced rather than decreased tumor vessel density (Fig. 1D), with the difference particularly apparent in the center (bottom panels, Fig. 1G) versus the invading edge of the tumors (top panels, Fig. 1G). These findings were neither unique to the U251 GBM cell line.
nor to the implantation of human cells into mice, because when mouse GBM cells (GL261) were intracranially implanted into the same animals used for the U251 cell studies, the mouse GL261 cells formed tumors of equal size in β3 WT and β3 KO animals (Fig. 1E) just as was noted in studies using human U251 GBM cells. Similarly, the GL261 tumors that formed in the β3 WT animals had a greater vessel density than those in the β3 KO animals (Fig. 1F). These results suggest that host β3 expression influences tumor behavior very differently depending on the site of tumor implantation.

Host β3 Expression Suppresses Intracranial Growth in a Bone Marrow-dependent Manner—One possible explanation for why host β3 expression enhanced intracranial tumor vascularity without increasing tumor size is that host β3 expression may have growth-suppressive actions as well as pro-growth, pro-angiogenic actions. A first clue as to what these growth suppressive actions might be was the observation that the intracranial GBM in β3 WT animals, in addition to being highly vascular, also had significantly more infiltration of F4/80-positive macrophages than was noted in the similarly sized, less vascular tumors in β3 KO animals (Fig. 2). Although macrophages are known to contribute to tumor elimination (31), our immunohistochemical analysis could not determine the relevance of the infiltrated F4/80-positive cells to tumor growth, the influence of β3 expression on macrophage function, or the origin of these cells. To begin to answer these questions we performed studies in which immunodeficient female Rag1 KO/β3 WT (or β3 KO) animals were lethally irradiated and transplanted with the bone marrow from genetically matched β3 WT or β3 KO male animals. Four weeks later U251 GBM cells were intracranially implanted into animals with reconstituted bone marrow, after which effects of β3 expression in the transferred bone marrow on tumor growth and vascularity were monitored. As shown in Fig. 3A, the size of intracranial tumors in β3 WT animals that did not undergo irradiation/BMT (bar 1) was no different from that in β3 WT animals that had their β3 WT bone marrow replaced with that from a matched β3 WT animal (bar 3). Consistent with data in Fig. 1C, these tumors were also of a similar size to those that grew in β3 KO animals, which received either no irradiation/BMT (bar 2) or were transplanted with β3 KO marrow (bar 6). The transplantation of β3 WT marrow into β3-deficient mice, however, greatly suppressed the growth of intracranially implanted cells (bar 5), whereas the replacement of β3 WT marrow with β3-deficient marrow greatly enhanced tumor growth (bar 4). These results suggest that in addition to exerting a pro-angiogenic, pro-growth effect, host β3 expression also exerts a previously unidentified anti-tumor effect mediated by factors transferable in the bone marrow.

Bone Marrow-derived Macrophages Suppress Intracranial Tumor Growth in a β3-dependent Manner—The β3 integrin status of the host bone marrow could potentially influence intracranial tumor growth in several ways. The function of marrow-derived B and T lymphocytes is known to be dependent on αVβ3 expression (32), and loss of β3 expression could block B and T lymphocytes function, thereby leading to enhanced glioma growth. All experiments done in this study, however, were done in Rag1 KO animals that lack B and T lymphocytes, eliminating a possible role for these cells.

The bone marrow has also been suggested to contribute cells to the growing vasculature of tumors (33, 34), and it is possible that host β3 expression could suppress tumor growth by alter-
Effects of Host αVβ3 Integrin Expression on Gliomas

FIGURE 3. Analysis of intracranial U251 GBM tumor size and vessel density after BMT. β3 WT or β3 KO recipient female mice were lethally irradiated and transplanted with β3 WT or β3 KO marrow isolated from male littermate donor mice. 4 weeks after BMT, U251 cells were injected intracranially. Panel A, 35 days after tumor implantation, animals were euthanized, the brain was sectioned coronally, and tumor volume was analyzed and expressed as a percentage of control (18 ± 4 mm³, β3+/− w/o BMT). Enhanced tumor growth was observed in only mice reconstituted with β3 KO marrow each group. Panel B, sections were stained with anti-wWF antibody, and wWF-positive vessels detected by a rhodamine-conjugated secondary antibody were counted in 10 independent tumor fields (HPF) at ×400 with the aid of an ocular grid. Tumor vessel density was higher in β3 WT animals than in β3 KO animals but was independent of the β3 status of the host BM. Results are expressed as the means ± S.D. * or **, p < 0.05.

ing neovascularization. The alterations in tumor growth mediated by BMT, however, were unrelated to vessel density, and as shown in Fig. 3B, tumor vessel density was related to β3 status of the host but was unrelated to β3 status of the host bone marrow. Consistent with this observation, no significant amounts of cells from the bone marrow of male mouse donors (as assessed by Y chromosome in situ hybridization) were found associated with the tumor vasculature (not shown), suggesting that the bone marrow did not contribute in a significant manner to the generation of tumor vasculature or to host β3 effects on blood vessel density.

A third possibility is that bone marrow-derived cells generated even in Rag1 KO mice (macrophages, NK cells, neutrophils) could directly or indirectly suppress intracranial tumor growth. Because more macrophages were found associated with intracranial tumors in β3 WT animals than in β3 KO animals, we first considered the possibility that β3 WT animals produced more tumor-suppressive macrophages than β3 KO animals. Examination of levels of circulating macrophages in β3 WT and β3 KO animals by flow cytometry, however, showed that levels of F4/80-positive cells were not significantly different in β3 WT and β3 KO animals (not shown). We, therefore, examined the extent of infiltration of F4/80-positive cells into the tumor-bearing hemisphere and the non-tumor-bearing hemisphere of β3 WT or β3 KO animals with or without BMT. In all animals examined, no F4/80-positive or bone marrow-derived Y chromosome-positive cells were found in the non-tumor-bearing hemisphere (not shown). As shown in Fig. 4, A and B, however, significant amounts of F4/80-positive cells were found in the tumor-bearing hemisphere of β3 WT mice that received β3 WT bone marrow, and >80% of these DAPI-positive (top left, Fig. 4C) and F4/80-positive (top right, Fig. 4C) cells were also Y chromosome-positive (bottom left, Fig. 4C) and, therefore, bone marrow-derived (merge photo, bottom right, Fig. 4C). The number of F4/80-positive, Y chromosome-positive cells in the tumor-bearing hemisphere of β3 WT mice with β3 WT marrow was no different from that in β3 KO mice that received β3 WT marrow but was significantly greater than that in β3 WT or β3 KO animals with β3 KO marrow (Fig. 4, A and B). The apparent inability of β3-deficient macrophages to reach the tumor was mirrored by alterations in the in vitro migration ability of these cells. In these studies, HDMEC were grown to confluence on laminin-precoated polycarbonate filters, after which macrophages isolated from β3 WT or β3 KO mice were seeded on top of the monolayer and assayed for their ability to transmigrate. As shown in Fig. 4D, β3 KO macrophages had less transmigration ability than β3 WT macrophages, and only the migratory ability of the β3 WT macrophages was inhibited by preincubation with anti-β3 integrin antibodies (Fig. 4D).

To further verify that β3 WT, bone marrow-derived macrophages were responsible for the glioma growth suppression noted in β3 WT animals, we used a macrophage-specific antibody to selectively deplete animals of macrophages, after which the effects on glioma growth were monitored. Treatment of β3 WT or β3 KO animals with a macrophage-selective antibody resulted in a >90% depletion of circulating macrophages in both groups (relative to circulating macrophage levels in animals treated with an IgG control) and nearly eliminated macrophage infiltration into tumors in both groups (bottom panel, Fig. 5) while not altering the level of circulating NK cells (not shown). Although this macrophage depletion did not significantly affect the growth of U251 cells intracranially implanted into β3 KO animals (Fig. 5, far right bars), macrophage depletion significantly increased the growth of U251 cells intracranially implanted into β3 WT animals (Fig. 5, first and second bars). As a whole, these results suggest that macrophages derived from β3 KO bone marrow can circulate in the bloodstream but appear to be defective in their ability to cross out of the vasculature and into the tumor and to suppress the growth of intracranially implanted GBM cells.

β3 WT Macrophages Secrete TNFα and Are Associated with Tumor Apoptosis—Although β3 WT macrophages migrated to intracranial tumors more effectively than β3 KO macrophages, the means by which they suppressed tumor growth once at the site remained unclear. Macrophage infiltration did not appear to slow the growth of tumor cells in a general fashion because the relatively smaller tumors that grew in presence of infiltrated β3 WT macrophages had a tumor growth fraction (Ki67 label-
Effects of Host αVβ3 Integrin Expression on Gliomas

FIGURE 4. Bone marrow-derived macrophages suppress intracranial tumor growth in a β3-dependent manner. Panels A and C, brain sections from lethally irradiated β3 WT or β3 KO female animals reconstituted with bone marrow from β3 WT or β3 KO male animals were stained with DAPI (as a measure of cellularity; blue) and subjected to immunohistochemistry (F4/80 antibody) and in situ hybridization (Y chromosome) with subsequent immunofluorescent detection of rhodamine and FITC-conjugated secondary antibodies (red and green, respectively). F4/80-positive/Y chromosome-positive cells (as in the merged images in panel C) were then counted in the GBM-bearing hemisphere and compared among groups (panel B). More F4/80-positive cells and more F4/80/Y chromosome double-positive cells were detected around the smaller tumors that arose in animals with transplanted β3 WT marrow (rows 1 and 3 of panel A and the dark bars of panel B) than in the larger tumors that arose in animals with transplanted β3 KO marrow (rows 2 and 4 of panel A and the light bars of panel B). Results are expressed as the means ± S.D. * and **, p < 0.05. Panel D, for in vitro transendothelial migration assays, freshly isolated macrophages from β3 WT or β3 KO animals (10^6 cells) were preincubated with control or anti-β3 integrin antibody (Ab) (50 μg/ml), seeded onto transwell plates covered with HDMEC cells, and measured for their ability to cross the endothelial cell layer in a 5-h period. β3 KO macrophages had less transmigration ability than β3 WT macrophages, and the migratory ability of the β3 WT macrophages was inhibited by preincubation with anti-β3 integrin antibodies. Results are expressed as the means ± S.D. * and **, p < 0.05.

ing index) no different from that of relatively larger tumors that grew in the absence of β3 WT macrophages (not shown). We, therefore, examined tumors from β3 WT or β3 KO animals bearing β3 WT or β3 KO bone marrow for extent of apoptosis. As shown in Fig. 6A, the relatively larger tumors that formed in animals with β3 KO bone marrow (β3 WT or β3KO hosts transplanted with β3 KO bone marrow) were devoid of TUNEL-positive apoptotic cells. In contrast, a significant number of TUNEL-positive apoptotic cells was present within the relatively smaller tumors that formed in animals with β3 WT bone marrow (β3 WT or β3KO hosts transplanted with β3 WT bone marrow). Apoptotic cells were particularly apparent at the invading edges of the tumor where the largest amount of bone marrow-derived F4/80-positive macrophages were also seen. Because macrophages can induce apoptosis by secretion of TNFα (31), we also immunohistochemically examined tumors from β3 WT and β3 KO animals bearing β3 WT or β3 KO bone marrow for the presence of TNFα-positive, F4/80-positive macrophages. As shown in Fig. 6B, although scattered F4/80-positive macrophages were found in the relatively larger tumors that formed in animals with β3 KO bone marrow, no double-positive TNFα-secreting macrophages were noted. Significant numbers of TNFα-secreting macrophages, however, were found in the invading, apoptotic regions of the relatively smaller tumors that formed in animals with β3 WT bone marrow. The apparent inability of macrophages from β3 KO animals to induce apoptosis was also mirrored by alterations in their in vitro TNFα secretion. In these studies macrophages isolated from β3 WT or β3 KO mice were cultured and allowed to secrete TNFα into the supernatant, after which the supernatants were added to U251 cells, and the extent of TNFα secretion and supernatant-induced U251 cell apoptosis (% of cells with a sub-G1 DNA content) were determined. As shown in the top panel of Fig. 7, β3 KO macrophages expressed less TNFα than β3 WT macrophages. The supernatant from β3 KO macrophages was also significantly less able to induce apoptosis in target U251 cells.
than similar supernatant from β3 WT macrophages (bottom panel, Fig. 7).

These results as a whole suggest that β3 WT macrophages have a greater ability to migrate to the tumor, secrete TNFα, and to participate in the apoptotic cell death in the tumor. These tumor-suppressive effects in turn appear to counter the enhanced vascular density stimulated by β3 integrins may play in glioma. In the present study we have shown that host αVβ3 integrin expression does play a role in glioma growth and angiogenesis but that this role is highly dependent on the microenvironment. We also show that host β3 expression has a variety of effects on glioma behavior in the intracranial setting, enhancing both tumor vascularity and growth while at the same time enhancing the ability of macrophages to extravasate, secrete TNFα, and participate in the suppression of glioma growth. These results suggest a more complex view of the function of host αVβ3 expression than previously described.

Although host β3 expression has been shown to play a critical and negative role in the angiogenesis and growth of a variety of implanted tumor cell types (14, 15), all published studies to date have been performed in the subcutaneous setting, leaving
open the question of how microenvironment influences integrin function. The data presented in this study both confirm previous work as well as highlight the effect of microenvironment on integrin β3 function. The present study shows that subcutaneously implanted GBM cells, like previously examined subcutaneously implanted melanoma and lung carcinoma cells (15), exhibit less tumor growth and less angiogenesis in β3 WT hosts than in β3 KO hosts. Surprisingly, however, the previously demonstrated anti-angiogenic effects of host β3 expression were reversed in the intracranial setting. The anti-angiogenic effects of host β3 expression in the subcutaneous setting were ascribed to up-regulation of VEGF receptor 2 that occurred in a compensatory fashion in β3 KO endothelial cells but not in β3 WT endothelial cells (35). In supplemental data, we confirmed that expression of VEGF receptor 2 is significantly higher in several tissues of β3 KO animals than in the same tissues from β3 WT animals. The brain, however, exhibited very low levels of VEGF receptor 2 expression, and these levels were not influenced by β3 expression. It is possible, therefore, that although endothelial cells in most microenvironments respond to the loss of β3 expression by up-regulating VEGF receptor 2 and increasing proliferation and blood vessel formation, brain endothelial cells are incapable of doing so and can, therefore, not initiate angiogenesis in response to loss of β3 expression. If this is the case, however, additional mechanisms must exist by which β3 expression rather than loss of β3 also enhances intracranial tumor vessel density.

In contrast to the tissue-specific differences in β3 effects on angiogenesis, the present studies suggest that β3 expression provides a microenvironment-independent, suppressive action on tumor growth. As in the subcutaneous setting, host β3 expression suppressed the growth of intracranial tumors, although this effect was masked by the pro-tumorigenic effects of host β3 expression on angiogenesis. Studies in the subcutaneous setting suggested that the anti-tumor effects of host β3 expression were mediated by bone marrow-derived cells (15), although the identity of these cells was not demonstrated. In the present study we clearly show that the ability of host β3 expression to suppress intracranial tumor growth was associated with the bone marrow and specifically with macrophages, which in β3 WT animals were able to extravasate and migrate into the tumor, where their presence was linked to TNFα secretion and tumor cell apoptosis. The function of αvβ3 in macrophages is not well defined, although integrins appear to be involved not only in the cell-cell contact required for macrophage extravasation and migration but also for the recognition and killing of tumor cells (30, 31). The reduced ability of β3 KO macrophages to cross a layer of endothelial cells in vitro as well as their inability to localize to the tumor in vivo suggests that the loss of β3 expression interferes with macrophage function at several levels although not with the production of the circulating macrophages themselves. Finally, although macrophage function was linked to the effects of β3 on tumor growth and macrophage depletion studies were shown to selectively deplete macrophages and not NK cells, the present studies cannot rule out the possibility that other bone marrow-derived cells such as neutrophils may also contribute to host β3 effects of glioma growth and angiogenesis. More detailed studies of the effects of these cells are likely to be of value.

Although the present work helps define the role of host β3 expression in the control of tumor growth and angiogenesis, it also has implications for tumor therapy. In the subcutaneous setting, β3 expression appears to contribute only to tumor suppression, and loss of β3 expression appears to only stimulate tumor growth and angiogenesis. In this type of setting there would appear to be little therapeutic value for a true β3 antagonist, although molecules that function as β3 agonists, as has been suggested for existing integrin-targeted therapeutics (36), would be predicted to be of value. In the more complex intracranial glioma setting, host β3 expression has a more mixed function. In the intracranial setting, β3 agonists could stimulate the elimination of the tumor by the innate immune system, although this might come at the cost of increased angiogenesis and growth of the tumor. β3 antagonists might
Effects of Host αVβ3 Integrin Expression on Gliomas

block tumor angiogenesis but would also suppress tumor elimination. It seems likely that appropriate use of well defined integrin targeting agents could minimize the growth-enhancing effects of the compounds while enhancing the growth suppressive effects of integrin expression. The identification of the effect of host β3 expression in the intracranial setting is a first step in this direction.

REFERENCES

1. Ludgate, C., Douglas, B. G., Dixon, P. F., Steinbok, P., Jackson, S. M., and Goodman, G. B. (1988) Int. J. Radiat. Oncol. Biol. Phys. 15, 1091–1095
2. Prados, M. D., Larson, D. A., Lamborn, K., McDermott, M. W., Sneed, P. K., Wara, W. M., Chang, S. M., Mack, E. E., Krouwer, H. G., Chandler, K. L., Warnick, R. E., Davis, R. L., Rabbitt, J. E., Malec, M., Levin, V. A., Gutin, P. H., Phillips, T. L., and Wilson, C. B. (1998) Int. J. Radiat. Oncol. Biol. Phys. 40, 57–63
3. CBTRUS (2000) Statistical Report: Primary Brain Tumors in the United States, 1992–1997, Central Brain Tumors Registry of the United States, Hinsdale, IL
4. Hynes, R. O. (2000) Cancer Res. 60, 2751–2758
5. Albeda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Altiparmak, D., and Ginsberg, M. H. (2002) Nat. Med. 8, 918–921
6. Prados, M. D., Larson, D. A., Lamborn, K., McDermott, M. W., Sneed, P. K., Wara, W. M., Chang, S. M., Mack, E. E., Krouwer, H. G., Chandler, K. L., Warnick, R. E., Davis, R. L., Rabbitt, J. E., Malec, M., Levin, V. A., Gutin, P. H., Phillips, T. L., and Wilson, C. B. (1998) Int. J. Radiat. Oncol. Biol. Phys. 40, 57–63