β1 Integrin Targeting Potentiates Antiangiogenic Therapy and Inhibits the Growth of Bevacizumab-Resistant Glioblastoma

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Abstract

Antiangiogenic therapies like bevacizumab offer promise for cancer treatment, but acquired resistance, which often includes an aggressive mesenchymal phenotype, can limit the use of these agents. Upregulation of β1 integrin (ITGB1) occurs in some bevacizumab-resistant glioblastomas (BRG) whereby, mediating tumor-microenvironment interactions, we hypothesized that it may mediate a mesenchymal-type resistance to antiangiogenic therapy. Immunostaining analyses of β1 integrin and its downstream effector kinase FAK revealed upregulation in 75% and 86% of BRGs, respectively, compared with pretreatment paired specimens. Furthermore, flow cytometry revealed eight-fold more β1 integrin in primary BRG cells compared with cells from bevacizumab-naive glioblastomas (BNG). Fluorescence recovery after photobleaching of cells engineered to express a β1-GFP fusion protein indicated that the mobile β1 integrin fraction was doubled, and half-life of β1 integrin turnover in focal adhesions was reduced markedly in BRG cells compared with bevacizumab-responsive glioblastoma multiforme cells. Hypoxia, which was increased with acquisition of bevacizumab resistance, was associated with increased β1 integrin expression in cultured BNG cells. BRGs displayed an aggressive mesenchymal-like phenotype in vitro. We found that growth of BRG xenograft tumors was attenuated by the β1 antibody, OS2966, allowing a 20-fold dose reduction of bevacizumab per cycle in this model. Intracranial delivery of OS2966 through osmotic pumps over 28 days increased tumor cell apoptosis, decreased tumor cell invasiveness, and blunted the mesenchymal morphology of tumor cells. We concluded that β1 integrin upregulation in BRGs likely reflects an onset of hypoxia caused by antiangiogenic therapy, and that β1 inhibition is well tolerated in vivo as a tractable strategy to disrupt resistance to this therapy. Cancer Res; 73(10): 3145–54. ©2013 AACR.

Introduction

Glioblastoma multiforme is the most common malignant brain tumor in adults, with a median survival close to 1 year (1). To date, only 3 drugs have been approved by the U.S. Food and Drug Administration for glioblastoma treatment, including, most recently, bevacizumab (Avastin; Genentech) in 2009 (2). Despite improvements in clinical metrics with other cancers treated with antiangiogenic therapy, the promise of antiangiogenesis in glioblastoma remains unfulfilled in part due to acquired resistance.

Integrins are cell-adhesion molecules that mechanosense the microenvironment and elicit extracellular-matrix (ECM)–induced signaling in both normal and pathologic states such as inflammation and cancer. Importantly, integrins lie at the interface of the cell and microenvironment, playing a key role in tumor progression and regulating growth and survival pathways. Upregulation of integrins has been associated with epithelial malignancies (5), particularly during invasion, metastasis, and angiogenesis (6, 7). There is growing evidence for the role of aberrantly expressed integrins in glioblastoma pathophysiology (8), β3 and β5 integrins have been implicated in angiogenesis, and several approaches targeting these molecules are under investigation in the clinic (9). β1 integrins, which coordinate much broader functional activities such as inflammation, proliferation, adhesion, and invasion, have recently been implicated in therapeutic resistance in multiple solid cancer models (10–13) and hematopoietic malignancies (14, 15). Importantly, this β1 integrin-mediated resistance is thought to occur at the level of the tumor cells themselves. β1 integrin also has important functions during tumor vascularization such as VEGF-dependent (16) and VEGF-independent angiogenesis by promoting endothelial cell migration (17).
Notably, as shown in glioblastoma models (18–20), we found that bevacizumab causes U87MG, a commonly studied glioblastoma cell line, to grow more invasively (Supplementary Fig. S1A), and orthotopic bevacizumab-resistant glioblastoma (BRG) xenografts infiltrate the brain via vessel co-option, which has been shown to require β1 integrins (21), whereas bevacizumab-naïve glioblastoma (BNG) xenografts remain well circumscribed (Supplementary Fig. S1B; ref. 22). This evidence, when taken together, led us to investigate the novel hypothesis that β1 integrin drives resistance to antiangiogenic therapy by promoting multiple mechanisms at the interface of tumor cells and the microenvironment.

Materials and Methods

Collection and analysis of human clinical specimens

Human specimens were obtained from the University of California, San Francisco (UCSF; San Francisco, CA) Brain Tumor Tissue Bank (22). Fluorescence immunohistochemistry of human paraffin-embedded tissues for β1 integrin (ab52971; Abcam), CA9 (Novus Biologicals), FAK (ab1803; Abcam), GFAP (ab4674; Abcam), and laminin (ab14055; Abcam) was conducted with either standard indirect or tyramide signal amplification (PerkinElmer) as described (21) following standard citrate buffer (Abcam) antigen retrieval.

Cells and cell lines

U87MG, MDA-MB-231, and SW-1080 cell lines were obtained and authenticated by American Type Culture Collection and passed in less than 6 months. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, nonessential amino acids (NEAA), and antibiotics. The bevacizumab-resistant primary glioblastoma cells, SF7796 and SF8106, and the bevacizumab-naïve primary glioblastoma cells, SF7996 and SF8244, were obtained and grown as described (22). Bevacizumab-resistant (BRG1, BRG2, and BRG3) and bevacizumab-naïve (N1, N2, and N3) cell lines were derived from fresh clinical resection specimens at UCSF as described (22) and were propagated in 50% to 50% DMEM/F-12 with 10% FBS, NEAA, and antibiotics. Primary SF8106 and SF7796 glioma cultures were transfected with the pFB-Neo Retroviral Vector (Agi-lent) containing a cDNA expressing the β1-GFP fusion protein (gift of Martin Humphries; University of Manchester, UK), followed by selection in 5 μg/mL geneticin (Gibco), as described (23). Experimental hypoxia was induced by growth in 1% oxygen as described (24).

Short hairpin RNA-mediated integrin knockdown

U87MG, BRG2, and BRG3 cells were infected with short hairpin RNA (shRNA) lentiviral particles (Santa Cruz Biotechnology) targeting β3 integrin (sc-63292-V), β1 integrin (sc-35674-V), or vector control (shCTRL; sc-108084) per manufacturer’s protocol. After 48 hours, subcultured cells were selected in 1 μg/mL puromycin for 1 week.

Flow cytometry

Cells were trypsinized in 0.25% trypsin with EDTA, counted, resuspended in PBS at 10^6 cells/mL, and kept on ice. Cell surface β1 integrin was labeled with anti-β1 integrin antibodies OS2966 (OncoSynergy, Inc.), CD29 (Clone TS-2/16, BioLegend), or CD29 (Clone K20, BD). Cell surface c-MET was detected with Clone 95106 monoclonal antibody (mAb; R&D Systems). Flow cytometry was conducted with a BD FACS Calibur II machine, and data were analyzed using FlowJo software (Tree Star, Inc.).

Western blot analysis

Western blots were conducted as described previously (25), using primary antibody to β1 integrin (Biolegend; Abcam) along with αV, α5, α4, and β3 antibodies (Cell Signaling).

Fluorescence recovery after photobleaching

SF8106/B1-GFP and SF7996/B1-GFP cells were seeded at 10,000 cells per well on chambered coverglass (Fisher), which had been coated overnight with 10 μg/mL fibronectin (Sigma-Aldrich). Individual cells were analyzed on a Zeiss LSM 510 NLO Meta confocal microscope. Target-bleach regions were normalized to background observational bleaching. Images were collected every 30 seconds until fluorescence recovery plateau. Fractional fluorescence recovery and half-time of recovery were calculated as described (26).

Dynamic in vitro assays and time-lapse microscopy

Adhesion assays were conducted as described (21) on plates coated with 10 μg/mL fibronectin (Sigma). Scratch assays were conducted as described (27) on plates coated with 10 μg/mL fibronectin. Cell spreading assays were conducted by plating 10^3 cells on a 30-mm dish coated with 10 μg/mL fibronectin. Time-lapse phase-contrast microscopy was conducted as described (21) with a Zeiss Observer Z1 inverted microscope with an AxioCam MRm and accompanying AxioVision 4.8 software. Briefly, 30-mm dishes were placed upon a heated stage adapter (Stable Z with heated lid, Biophtechs), and images were captured every 90 to 180 seconds for up to 24 hours. Media were covered with sterile embryo-tested mineral oil (Sigma).

Cell adhesion was quantified as described (21). Cell spreading was quantitated as cell area (measured hourly) and percentage of spread cells per high-power field. For cell area, all whole cells in a ×40 field were measured manually in ImageJ (NIH) at time zero and every hour for 5 hours. Means were calculated at each time point and compared. The percentage of spread cells per ×40 field was similarly calculated hourly (not including time zero) for 12 hours. Cell migration and dendriticity factor for cell shape were measured as described (22, 28).

Cell proliferation

Bromodeoxyuridine (BrdU) immunofluorescence was conducted by incubating cells in 10 μmol/L BrdU (Sigma) for 1 hour in complete media followed by fixation in 1% paraformaldehyde. Cells were permeated in 0.1% Triton-X100 (Sigma) in PBS on ice for 5 minutes followed by incubation in biotinylated sheep anti-BrdU antibody (ab2284, Abcam) overnight at 4°C. Following PBS washes, cells were incubated with streptavidin-Alexa555 (Invitrogen) and coverslipped with
VectaShield mounting medium with DAPI (VectorLabs). BrdU-positive cells per high-power field were counted in three 40× fields per well, 3 wells per experiment with repeats.

Tumor spheroid assays
Aggregation assays were conducted by suspending tumor cells in PBS on ice for 15 minutes. To visualize β1 integrin, cells were incubated with Alexa 647 conjugated anti-CD29 (clone TS-2/16) for 15 minutes, spun down, and rinsed. Cell growth in acidified media was conducted as described (29). Spheroidal growth assays were conducted in 6-well ultra-low adhesion plates (CoStar) with standard conditions for growth of neurospheres (29) for 3 to 5 days. Spheroid dissolution assays were conducted by plating spheroids on culture dishes coated with 10 μg/mL of bovine serum albumin (BSA) control, fibronectin, laminin, or collagen type IV (all ECM from Sigma) for 5 to 7 days.

Xenograft studies
Animal procedures were approved by the UCSF Institutional Animal Care and Use Committee (IACUC). A total of 10³ to 10⁶ cells were injected s.c. or 10⁵ cells were injected stereotactically into the right caudate (orthotopic model) of 5- to 8-week-old female athymic mice. Mice were randomized stereotactically into the right caudate (orthotopic model) of 5- to 8-week-old female athymic mice. Mice were random-
ized into treatment groups after tumors were established (mean s.c. volume = 40–60 mm³ or 10 days after intracranial implantation). Bevacizumab and human immunoglobulin G (IgG) control (Sigma) were administered at 10 mg/kg intraperitoneally (i.p.) twice weekly. Inhibitory anti-β1 integrin mAb OS2966 or control IgG was administered at 1, 5, or 10 mg/kg i.p. twice weekly. For orthotopic studies, OS2966 or IgG (160 μg total) was delivered intratumorally over 28 days via rigid cannula connected to Model 1004 osmotic pumps (Alzet) implanted s.c. between the scapulae. Subcutaneous tumors were measured with calipers 1 to 2 times weekly, with volume calculated as described (24). Treatment continued until mice had to be sacrificed per IACUC criteria (e.g., 2.1-cm maximal dimension, cutaneous ulceration, or symptoms) or study end.

Tissue preparation and immunohistochemistry
Mice were sacrificed and tissue collected and prepared as described (21). Cryostat sections (20–30 μm in size) were collected and mounted directly on slides and were processed for immunohistochemistry as described (21). For TUNEL staining, the TMR red In Situ Cell Death Detection Kit (Roche) was used. Immunohistochemistry was conducted using antibodies against β1 (Abcam), laminin (Abcam), GFAP (Millipore), and GLUT-1 (Millipore). Collagen IV immunostaining was described previously (25).

Statistical analyses
Student t test was used for parametric comparisons between paired variables. The Mann–Whitney U test was used for nonparametric pairwise comparisons. Multivariate analyses were done with ANOVA with post hoc Dunnett (most analyses) or Bonferroni tests (e.g., xenograft studies) as appropriate. P < 0.05 was deemed statistically significant.

Results
Upregulation of β1 integrin after acquired resistance to bevacizumab
We recently reported by microarray analysis and confirmatory PCR that transcription of β1 integrin was upregulated in glioblastomas exhibiting the nonenhancing infiltrative pattern of growth that is viewed as unique to bevacizumab resistance (22). To further verify this result, we conducted immunohis-

tochemistry on BRGs and their paired pretreatment specimens and found increased β1 integrin in 9 of 12 cases (75%, Fig. 1A). This increased immunoreactivity was observed in both angio-
genic blood vessels (Fig. 1A, arrows) and tumor cells (Fig. 1B). Consistent with bona fide integrin signaling, we found increased phosphorylated focal adhesion kinase staining in tumor cells in 6 of 7 BRG cases compared with pretreatment, with staining tending to be close to ECM (Fig. 1C; refs. 30, 31). Furthermore, β1 integrin was increased in primary cells from BRG clinical specimens compared with primary cultures established from BNG specimens by fluorescence-activated cell sorting (P < 0.05; Fig. 1D). To rule out contributions from patient variability among clinically derived samples that could affect integrin expression, we experimentally derived a bevacizumab-resistant xenograft from U87MG (U87-BEV; ref. 25). Screening of integrin expression revealed that U87-BEV showed significantly higher expression of β1 integrin, along with αV and α5 integrins, 2 potential β1 heterodimer partners, which interact to promote fibronectin binding (32), compared with U87-IGG, a control line treated only with IgG (Supple-
mentary Fig. S2A and S2B).

Because the percentage of integrin that is mobile and the turnover of this mobile integrin in focal adhesions sustains integrin signaling, we engineered BRG and BNG cells to express a β1-GFP fusion protein and used fluorescence recovery after photobleaching (FRAP) to measure integrin turnover in focal adhesions (26). We found 2-fold faster β1 integrin turnover in focal adhesions and double the mobile fraction of β1 integrin in bevacizumab-resistant SF8106 cells than in bevacizumab-responsive SF7996 cells (Fig. 1E and Supplementary Fig. S2C). Thus, compared with BNG cells, bevacizumab-resistant cells exhibited more mobile β1 integrin, reflective of reduced cellular membrane barriers to β1 integrin mobility in these cells and more rapid turnover of β1 integrin in focal adhesions.

To verify the functional relevance of increased β1 integrin in glioblastoma cells, we conducted cell adhesion assays and found significantly greater adhesion to ECM in BRGs compared with classic glioblastoma lines and even the highly adhesive MDA-MB-231 human breast adenocarcinoma, which served as a positive control (Fig. 1F). Thus, the β1 integrin subunit is functionally upregulated in glioblastoma after acquired bevacizumab resistance.

Hypoxia associated with high β1 integrin expression in glioblastoma cells
Hypoxia is a common cellular stress, particularly in the setting of fast-growing cancers and variably during the course of antiangiogenic therapy. Hypoxia upregulates β1 integrin expression in the setting of wound healing via hypoxia-inducible factor-1α (HIF-1α)–stimulated β1 transcription
We showed nearly 80% more CA9 staining after bevacizumab resistance as compared with before, with areas of CA9 staining corresponding with high β1 integrin expression in patient glioblastoma specimens (Supplementary Fig. S3A). To verify that antiangiogenic therapy acutely increases β1 integrin expression in growing tumors in vivo, we stained glioblastoma tumors from mice taken within days of the last bevacizumab treatment. Striking β1 integrin immunoreactivity was observed in the treated tumors compared with controls, particularly in the hypoxic tumor core (Supplementary Fig. S3B). To confirm this mechanism, we cultured U87MG cells for 6 to 48 hours in 1% oxygen to simulate microenvironmental hypoxia. We observed a significant increase in β1 integrin expression in glioma cells in vitro (Supplementary Fig. S3C). This was a rapid and reversible cellular response, which we also showed in primary human glioblastoma cells and breast and colorectal carcinoma cells (Supplementary Fig. S3D). Therefore it is likely that hypoxia contributes to β1 integrin upregulation during bevacizumab therapy, thus promoting mechanisms of survival and evasion.

β1 integrin inhibition attenuates mesenchymal phenotype and function of BRG cells

To further establish a functional role for β1 integrin in the malignant phenotype of bevacizumab glioblastoma cells, we established several clones of the BRG3 line expressing shRNA-targeting β1 integrin (shBETA1) and shRNA-targeting control sequences (shCTRL; Supplementary Fig. S4A–S4D). An
epithelioid morphologic shift, including polygonal shape and tendency for growth in sheets, was observed in the shBETA1 clones compared with shCTRL cells (Supplementary Fig. S5A). Indeed, individual cell size as measured by cell area was significantly increased in the shBETA1 clones compared with shCTRL (Supplementary Fig. S5B). In addition, dendritic factor, a unitless measure of cell shape, confirmed mesenchymal morphology in the shCTRL cells compared with more epithelioid morphology in the shBETA1 clones (Supplementary Fig. S5C). To verify functional impairment of mesenchymal phenotype, we examined the knockdown cells in various static and dynamic in vitro assays. As hypothesized, BRG3-shBETA1 clones showed significantly decreased capacity for mesenchymal functions, including adhesion (Fig. 2A), cell spreading (Fig. 2B and C and Supplementary Movie S1), and migration (Fig. 2D and E and Supplementary Movie S2) on ECM compared with BRG3-shCTRL cells. Furthermore, bevacizumab-naive cells showed epithelial-like migration with broad lamellipodia (Supplementary Movie S3) in contrast with the saltatory mesenchymal-type migration seen in the BRG3 line. In addition, in vitro proliferation (Fig. 2F) was significantly attenuated in the BRG3-shBETA1 clones compared with BRG3-shCTRL cells. This effect of β1 integrin knockdown in the BRG3 line on cell growth in vitro was determined to be cytostatic, rather than cytotoxic (Supplementary Fig. S5D). Similar results on adhesion, migration, and proliferation were found when the BRG3 line was treated with the inhibitory β1 integrin mAb, OS2966 (Supplementary Fig. S6A and S6B and Supplementary Movie S4). Thus, β1 integrin is important for multiple tumor cell functions implicated in antiangiogenic therapy evasion, including persistent proliferation and mesenchymal phenotype.

**Spheroidal growth is impaired by β1 integrin inhibition**

Microenvironmental hypoxia (34) and acidification (29) are suspected consequences of antiangiogenic therapy, and both activate HIF-1α and HIF-2α expression. Both hypoxia-associated factors have also been linked to malignant stem-like phenotypes in glioblastoma cells including in vitro spheroid formation. Indeed, freshly isolated bevacizumab-resistant U87-BEV cells are more highly prone to spheroid formation in vitro compared with the control U87-IGG line under normal culture conditions (Supplementary Fig. S7A). Implantation of

![Figure 2. β1 integrin knockdown in resistant glioblastoma cells inhibits mesenchymal function.](#) β1 integrin knockdown clones are impaired in cell adhesion (A; *P < 0.01, ANOVA with post hoc Dunnett test), dynamic cell spreading (B; †; P < 0.05, ‡; P < 0.01, ANOVA with post hoc Dunnett test), and percentage of spread cells (C; †; P < 0.01, Mann–Whitney test) on fibronectin. D and E, dynamic time-lapse analysis in a scratch wound assay shows impaired migration in knockdown clones versus control (†; P < 0.05, ANOVA with post hoc Dunnett test). β1 integrin knockdowns showed decreased proliferation as shown by BrdU immunofluorescence (E; †; P < 0.01, t test). Scale bar, 120 µm. Data are mean ± SD. See also Supplementary Figs. S3 to S5.)


β1 integrin inhibition prevents BRG growth in vivo

To test the role of β1 integrin in glioma growth and bevacizumab resistance in vivo, we used several glioma cell models athymic mice. First, we showed attenuation of tumor growth in the BRG2 and BRG3 lines after stable knockdown of β1 integrin (polyclonal, approximately 70% knockdown). In both cases, tumor growth was significantly impaired in the shBETA1 cells compared with shCTRL cells (Fig. 3C). The same result was found in knockdown clones from the clinically derived BRG3 line (Fig. 3D). Furthermore, confirming the role of β1 integrins in spheroidal maintenance, we showed dissolution of glioblastoma spheroids when plated on classic β1 integrin ECM substrates, particularly fibronectin (Fig. 3E). Thus, the β1 integrin subunit is intimately involved in cell–cell interactions and malignant spheroidal growth in glioma cells in vitro, a phenotype that has been associated with increased malignancy in vivo.

glioblastoma spheroids has been shown to be associated with decreased survival in orthotopic xenograft studies compared with control cells (29). Importantly, we observed that acutely dissociated BRG1 and BRG3 glioblastoma cells in suspension show the highest β1 integrin expression at cell–cell contacts (Fig. 3A). Therefore, to investigate a potential role for β1 integrins in glioblastoma spheroid formation, we cultured U87MG cells under acidic stress. Spheroid formation in acidic media was attenuated in U87-shBETA1 (Supplementary Fig. S7B) cells compared with control U87-shCTRL cells (Fig. 3B). Similar results were obtained with parental U87MG cells treated with an inhibitory β1 integrin antibody (Supplementary Fig. S7C). To more directly study spheroid formation, we grew glioblastoma cells in suspension in nonadherent plates. U87 cells with stable knockdown of β1 integrin (U87-shBETA1) showed significantly smaller spheroid diameters than control U87-shCTRL cells (Fig. 3C). The same result was found in knockdown clones from the clinically derived BRG3 line (Fig. 3D).

In both BRG and BRG3 lines, we showed a significant decrease in cell contact and invasion capacity after knockdown of β1 integrin expression at cell contacts in 2 BRG lines in an in vitro aggregation assay. Scale bars, 30 and 10 μm, respectively. B, β1 knockdown in U87MG attenuates spheroid formation (arrows) in acidic media. Scale bar, 120 μm. Formal spheroidal growth assay confirms impairment of spheroid diameter with β1 knockdown in both U87MG (C; *, P < 0.05, t test; scale bar, 120 μm) and BRG3 clones (D; *, P < 0.05, ANOVA with post-hoc Dunnett test; scale bar, 120 μm). E, U87MG spheroids unravel when plated on fibronectin, but not BSA control substrate consistent with a β1-mediated mechanism. Scale bar, 120 μm. Data are mean ± SD. See also Supplementary Fig. S6.

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To provide proof-of-concept for a clinically relevant treatment modality, we tested a therapeutic antibody approach in a glioma xenograft model. Mice inoculated with the BRG3 cell line were systemically treated with inhibitory β1 integrin monoclonal antibodies (OS2966) at 2 doses. Both groups of treated mice had significantly smaller tumors than mice treated with 5 mg/kg of control IgG (Fig. 4D and E). Strikingly, 56% (5/9) of mice with BRG3 tumors treated with 5 mg/kg OS2966 showed complete tumor regression after 8 weeks of treatment. We observed apoptosis on histology in the treated group but not in the IgG control (Fig. 4F). Finally, to verify the above findings in an orthotopic model, we stereotactically implanted BRG3 cells into the striatum of mice. Ten days after inoculation, we implanted cannulae through the same burr hole for chronic intratumoral delivery of either OS2966 or IgG control via s.c. osmotic pumps that provided antibody delivery for 28 days. Successful tumor delivery of antibodies was verified with immunofluorescence detection of OS2966 in horizontal brain sections (Supplementary Fig. S8). BRG3 cells in the control condition formed highly invasive tumors, and 55.2% ± 11% of invading cells were associated with the neurovasculature according to GLUT-1 immunoreactivity for

Figure 3. β1 knockdown in glioma cells inhibits spheroidal growth. A, immunofluorescence shows acutely enriched β1 immunoreactivity at cell–cell contacts in 2 BRG lines in an in vitro aggregation assay. Scale bars, 30 and 10 μm, respectively. B, β1 knockdown in U87MG attenuates spheroid formation (arrows) in acidic media. Scale bar, 120 μm. Formal spheroidal growth assay confirms impairment of spheroid diameter with β1 knockdown in both U87MG (C; *, P < 0.05, t test; scale bar, 120 μm) and BRG3 clones (D; *, P < 0.05, ANOVA with post-hoc Dunnett test; scale bar, 120 μm). E, U87MG spheroids unravel when plated on fibronectin, but not BSA control substrate consistent with a β1-mediated mechanism. Scale bar, 120 μm. Data are mean ± SD. See also Supplementary Fig. S6.
endothelial cells (Fig. 5A). OS2966 treatment resulted in complete regression of tumors (3/3 mice) as supported by massive induction of tumor cell apoptosis on TUNEL staining (Fig. 5B). Finally, OS2966 treatment inhibited striatal invasion as shown by number of invading cells per section (Fig. 5C) and invasion distance (Fig. 5D). Accordingly, OS2966 treatment seemed to directly affect invasive morphology of tumor cells (Fig. 5E).

Thus, blockade of $\beta_1$ integrin function with inhibitory monoclonal antibodies attenuates growth of a recurrent bevacizumab-resistant glioma line in vivo. $\beta_1$ integrin inhibition potentiates bevacizumab treatment

Not all patients respond to bevacizumab, and significant morbidity is associated with its long-term use. Therefore, there is clinical need for a novel therapeutic approach to improve response rates, prevent resistance, and potentially reduce the necessary dose of bevacizumab. Toward this end, full-dose combination therapy of OS2966 with bevacizumab was attempted in the U87MG subcutaneous xenograft model in 2 separate investigations. However, both studies had to be halted before their endpoints due to rapid cutaneous tumor ulceration in 100% (5/5 and 10/10 mice, respectively) of the combination therapy animals by the third week of treatment. These findings are suggestive of synergy between OS2966 and bevacizumab. To explore this question further, we hypothesized that the addition of OS2966 alternating with low-dose bevacizumab would enhance efficacy in U87MG xenografts (Fig. 6A). Indeed, alternating OS2966 and bevacizumab at 1 mg/kg, each was equivalent to a biweekly standard dose of bevacizumab (10 mg/kg) in this setting (Fig. 6B). Furthermore, the percentage of animals with complete regression of tumors was nearly equivalent, with 44% (4/9) of mice in the alternating low-dose group compared with 56% (5/9) of mice in the standard dose group. Thus, consistent with its multiple roles in promoting therapeutic resistance, $\beta_1$ integrin is potentially a promising adjunct target in combination with antiangiogenic therapies, including bevacizumab.
Discussion

Our data show for the first time that resistance to antiangiogenic therapy is associated with increased expression of β1 integrin as well as increased mobility of β1 integrin and increased turnover of β1 integrin in focal adhesions. We also showed that resistance to antiangiogenic therapy can be overcome by targeting tumor cell interactions with the glioma microenvironment via β1 integrin inhibition. In contrast to approaches that target a specific integrin–ligand interaction (e.g., natalizumab, which targets α4β1 integrin, blocking the ability of α4β1 or α4β7 to bind VCAM-1), a single drug targeting β1 would inhibit the ability of tumor cells to bind a spectrum of ECM ligands like fibronectin, collagen IV, and laminin, each of which we found to be upregulated in BRGs (22). This approach could potentially be applied more broadly in several medical indications. Inhibition of β1 integrin overcomes resistance to antiangiogenesis therapy via multiple potential mechanisms: (i) preventing vessel cooption (and/or growth/invasion upon any classical ECM substrate; ref. 21); (ii) reducing viability of tumor cells after insults (35); (ii) directly inhibiting tumor cell proliferation; (iii) directly inhibiting the vascularization process (36); and (iv) inhibiting the aggressive mesenchymal phenotype, including spheroidal growth. In addition, β1 integrin inhibition alternating with bevacizumab can reduce the necessary dose of antiangiogenic agent, thus potentially reducing drug-related morbidity.

It is tempting to speculate that because breast and other cancer cells also respond to insults with an increase in β1 integrin expression (e.g., Supplementary Fig. S3D), this approach may be applicable for the treatment of a wide variety of high-grade and/or treatment-refractory adenocarcinomas. This hypothesis warrants further study. Indeed, β1 integrin has proved to be a viable oncological target in animal models of solid and blood-borne cancers for multiple processes, including (i) prevention and growth of metastasis (37); (ii) overcoming resistance to radiation (35) and chemotherapy (38–42); and (iii) inhibition of local carcinoma spread. In addition, β1 integrin upregulation has been shown to be prognostic for breast (38, 43), lung (44), and pancreatic (37) cancers.

A limitation of the current study is the inability to directly study the effect of antiangiogenesis mechanistically in vitro. Bevacizumab acts at the endothelium, causing ischemia and other tissue-level processes, which cannot be accurately modeled with isolated tumor cells or cocultures. Therefore, the
spectrum of adaptations to antiangiogenic therapy cannot currently be reliably assessed \textit{in vitro}. We have addressed this limitation with a multimodal approach using clinical specimens and their cellular derivatives in surrogate \textit{in vitro} assays (e.g., hypoxia, acidified media) and relevant preclinical murine models. Of note, these murine models were generated by treating tumors with bevacizumab, which targets human, not murine VEGF, and would therefore target only tumor cells rather than stromal cells. However, we have found that bevacizumab is equally effective in xenograft treatment as an agent-targeting mouse and human VEGF (personal communication, C. David James, Department of Neurological Surgery, University of California, San Francisco, CA; 2008).

The orthotopic model with bevacizumab-resistant BRG3 cells revealed several important findings. First, we confirmed that parenchymal invasion of bevacizumab-resistant glioma cells occurs primarily via vessel cooption. Second, OS2966 delivered via osmotic pump nearly completely abolished invasion of BRG3 cells. In addition, inhibition of β1 integrin in these studies resulted in tumor cell apoptosis and disrupted tumor cell growth with low-dose combo (group II) was equivalent to high-dose bevacizumab (group I) compared with IgG controls in subcutaneous U87MG xenografts (\(P < 0.05\), ANOVA with post hoc Bonferroni test). Shown are mean ± SEM.

| Figure 6. | OS2966 potentiates efficacy of bevacizumab in a glioma model. A, study design of experimental groups with standard bevacizumab therapy at 10 mg/kg biweekly (group I, BEV) versus alternating bevacizumab and OS2966 at 1 mg/kg (group II, low-dose combo). B, inhibition of tumor growth with low-dose combo (group II) was equivalent to high-dose bevacizumab (group I) compared with IgG controls in subcutaneous U87MG xenografts (\(P < 0.05\), ANOVA with post hoc Bonferroni test). Shown are mean ± SEM. |

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