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Integrin control of the transforming growth factor-β pathway in glioblastoma

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Transforming growth factor-β is a central mediator of the malignant phenotype of glioblastoma, the most common and malignant form of intrinsic brain tumours. Transforming growth factor-β promotes invasiveness and angiogenesis, maintains cancer cell stemness and induces profound immunosuppression in the host. Integrins regulate cellular adhesion and transmit signals important for cell survival, proliferation, differentiation and motility, and may be involved in the activation of transforming growth factor-β. We report that αvβ3, αvβ5 and αvβ8 integrins are broadly expressed not only in glioblastoma blood vessels but also in tumour cells. Exposure to αv, β3 or β5 neutralizing antibodies, RNA interference-mediated integrin gene silencing or pharmacological integrin inhibition using the cyclic RGD peptide EMD 121974 (cilengitide) results in reduced phosphorylation of Smad2 in most glioma cell lines, including glioma-initiating cell lines and reduced transforming growth factor-β-mediated reporter gene activity, coinciding with reduced transforming growth factor-β protein levels in the supernatant. Time course experiments indicated that the loss of transforming growth factor-β bioactivity due to integrin inhibition likely results from two distinct mechanisms: an early effect on activation of preformed inactive protein, and second, major effect on transforming growth factor-β gene transcription as confirmed by decreased activity of the transforming growth factor-β gene promoter and decreased transforming growth factor-β_1 and transforming growth factor-β_2 messenger RNA expression levels. *In vivo*, EMD 121974 (cilengitide), which is currently in late clinical development as an antiangiogenic agent in newly diagnosed glioblastoma, was a weak antagonist of pSmad2 phosphorylation. These results validate integrin inhibition as a promising strategy not only to inhibit angiogenesis, but also to block transforming growth factor-β-controlled features of malignancy including invasiveness, stemness and immunosuppression in human glioblastoma.

Keywords: glioma; integrin; TGF-β; cilengitide

Abbreviation: TGF = transforming growth factor

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Introduction

Human glioblastoma is a highly lethal brain tumour that kills affected patients by local destructive growth. Surgery, radiotherapy and alkylating agent chemotherapy represent the current standard of care, which results in a median overall survival of <1 year on a population level (Preusser *et al*., 2011). Transforming growth factor (TGF)-β is a master molecule controlling migration, invasiveness and angiogenesis in glioblastoma and induces profound immunosuppression (Wick *et al*., 2006). More recently, TGF-β has also been attributed to an important role in maintaining the population of cells within glioblastomas exhibiting stem cell–like properties, often (and also herein) referred to as glioma-initiating cells (Penuelas *et al*., 2009; Anido *et al*., 2010). Rodent glioma models have confirmed TGF-β as an attractive target for experimental approaches using gene silencing or small-molecule TGF-β receptor antagonists (Friese *et al*., 2004; Uhl *et al*., 2004; Zhang *et al*., 2011). Clinical data confirm that TGF-β/Smad pathway activity confers a poor prognosis to glioma patients (Bruna *et al*., 2007), and TGF-β antagonism is currently explored as a therapeutic strategy in recurrent glioblastoma (Bogdahn *et al*., 2011; Azaro *et al*., 2012). However, although the important role of TGF-β in glioblastoma and other cancers is widely accepted, its therapeutic targeting has remained challenging.

TGF-β is secreted in a latent complex with its processed propeptide, latency-associated peptide (Annes *et al*., 2003) in the small latent complex. Latent TGF-β-binding proteins covalently bind the small latent complex to form the large latent complex (Miyazono *et al*., 1991). Activation of TGF-β necessitates dissociation from the small latent complex, which involves proteolytic cascades or cell traction forces (Wipff and Hinz, 2008). Integrins constitute a large family of cell surface transmembrane molecules that are composed of an α- and a β-subunit. Eighteen α-subunits and β-subunits form 24 different heterodimeric integrin molecules, which mediate various complex processes including adhesion, migration and angiogenesis in a cell type– and context-dependent manner (Desgrosellier and Cheresh, 2010). Importantly, integrins may also play an important, but complex, role in activating TGF-β (Sheppard, 2005; Wipff and Hinz, 2008). Diverse integrins, including all αv integrins, interact with the latency-associated peptide/TGF-β_1 portion of the large latent complex via a L-arginine, glycine, and L-aspartic acid (RGD) motif (Ludbrook *et al*., 2003; Sheppard, 2005) and αvβ6 and αvβ3 may support TGF-β_1 activation (Munger *et al*., 1999; Mu *et al*., 2002). However, TGF-β_2/latency-associated peptide does not contain an RGD sequence, and whether TGF-β_2 activation depends on other integrin-dependent mechanisms remains unclear (Sheppard, 2005). Additionally, TGF-β_1 transcription and secretion are stimulated through integrin activation via an Arg-Gly-Asp-Ser (RGDS) peptide in human mesangial cells (Ortega-Velazquez *et al*., 2003). Taken together, these observations prompted us to explore the possibility of down regulating the TGF-β pathway in glioblastoma by interfering with the expression or function of specific integrins expressed in these tumours.

Materials and methods

Cells and reagents

The human malignant glioma cell lines U87MG and T98G were from the American Type Culture Collection. LN-18, LNT-229 and LN-308 cells were kindly provided by N. de Tribolet (Lausanne, Switzerland). The generation, characterization and culturing of glioma-initiating cell lines GS-2, GS-3, GS-5 and GS-9 have been described previously (Gunther *et al*., 2008). These cells were maintained as sphere cultures. Transfected mink lung epithelial cells, a kind gift from D.B. Rifkin (New York, NY), were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (Biochrom) supplemented with G418 (800 μg/ml, Biochrom). Accutase™ was obtained from PAA. Recombinant human TGF-β_1 was purchased from R&D Systems. Except for immunohistochemistry, the following antibodies were used for all experiments: rabbit polyclonal antibody to TGF-β_2 and goat polyclonal antibody to actin were obtained from Santa Cruz Biotechnology. TGF-β_1, phospho-Smad2 (Ser465/467) (clone 138D4) and Smad2 (clone 86F7) antibodies were purchased from Cell Signaling Technology. Mouse anti-Smad2/3 antibody was from BD Biosciences. Anti-αv antibody (clone 17E6) was obtained from Merck. The antibodies to αvβ3 (clone LM609, MAB1976), αvβ5 (clone P1F6, MAB1961) and to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Chemicon. Mouse serum-free hybridoma supernatant to integrin β8 (Ab14e5), a generous gift from S.L. Nishimura (San Francisco, CA), was used at a dilution of 1:100 for flow cytometry. Murine isotype mouse IgG_1 from murine myeloma was purchased from Sigma-Aldrich. The TGF-β receptor I kinase inhibitor SD-208 was kindly provided by Scios Inc. Cilengitide was provided by Merck (Darmstadt, Germany). For transient transfections, 3×10^5 glioma cells were seeded in a six-well plate and transfected with 100 nM of small interfering RNA pools against a specific integrin or irrelevant scrambled control. For all integrins, two different small interfering RNA pools were purchased (Dharmacon/Thermo), using Metafectene® Pro (Biontex).

Real-time polymerase chain reaction

Total RNA was prepared using the RNeasy® system including DNase treatment (Qiagen) and transcribed according to standard protocols. Complementary DNA was prepared using SuperScript® RNase H^- Reverse Transcriptase (Invitrogen) and random nonamers (Cell Signaling). For real-time PCR, gene expression was measured in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with SYBR Green Master Mix (Eurogentec) and primers (Sigma-Aldrich) at optimized concentrations. GAPDH primers have been previously described (Carraro *et al*., 2005). The other sequences were as follows: TGF-β_1, forward 5′-GCCCTGGACACCAACTATTG-3′ [NM_000660.3, nucleotides (nt) 1702–1727], reverse 5′-CGTGTCCAGGCTCCAAATG-3′ (NM_000660.3, nt 1851–1869); TGF-β_2, forward 5′-AAGCTTACACTGTCCCTGCTGC-3′ (NM_003238.1, nt 847–868), reverse 5′-TGTGGAGGTGCCAT CAATACCT-3′ (NM_003228.1, nt 934–955); integrin αv, forward 5′-AAGTAAGCCCAGTTGTATCTCACAA-3′ (NM_002210, nt 2529–2554), reverse 5′-GGACTCGAGACTCCTCTTATCTCAA-3′ (NM_002210, nt 2581–2605); integrin β3, forward 5′-CAGATGC CTGCACCTTTAAGAAA-3′ (NM_000212, nt 1912–1934), reverse 5′-TCACGGCAGTAACGGTTGC-3′ (NM_000212, nt 1990–2008); integrin β5, forward 5′-CTACACGGCACCGAGGTACC-3′ (NM_002213, nt 870–889), reverse 5′-CGCAATTTGGAAACAACTT

GTAAC-3′ (NM_002213, nt 908–931); integrin β8, forward 5′-TGAAAGTCATATCGGATGGCG-3′ (NM_002214, nt 1503–1523), reverse 5′-GCACCACTATGCCTGCCAAT-3′ (NM_002214, nt 1590–1609). The conditions were 40 cycles at 95°C/15 s and 60°C/1 min. Standard curves were generated for each gene and the amplification was 90–100% efficient. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to GAPDH and calculated with the ΔC_{TT} method for relative quantification.

Immunoblot analysis

For the detection of proteins from cell lysates, cells were lysed in radioimmunoprecipitation assay buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) supplemented with 1 × complete inhibitor mix (Roche Diagnostics) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich). Serum-free conditioned media were concentrated with the centriplus centrifugal filter device YM-3 (3000 Da cut-off; Millipore). Protein levels were analysed by immunoblot using 20 μg of protein per lane mixed with Laemmli buffer containing β-mercaptoethanol, unless otherwise indicated, with the respective antibodies used at the concentrations recommended by the manufacturer. Equal protein loading was ascertained by Ponceau S staining. Visualization of protein bands was accomplished using horseradish peroxidise–coupled secondary antibodies (Sigma-Aldrich) and enhanced chemiluminescence (Perbio).

Flow cytometry

Glioma cells were detached using Accutase™ (PAA), resuspended in buffer for flow cytometry (PBS, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bovine serum albumin) and incubated for 30 min on ice using the respective antibodies or isotype-matched control antibody. This was followed by an R-phycoerythrin-conjugated goat anti-mouse IgG antibody (1:40) labelling step. Fluorescence was detected in a CyanADP flow cytometer (DakoCytomation). Specific fluorescence indices were calculated by dividing the mean fluorescence obtained with the specific antibody by the mean fluorescence obtained with the control antibody.

TGF-$\beta_{1/2}$ enzyme-linked immunosorbent assay

The cells (2×10^5/well) were seeded in a six-well plate and exposed to the control peptide RAD or cilengitide as indicated. Supernatants were harvested after 48 h. To activate latent TGF-$\beta_{1/2}$ to the bioactive form, hydrochloric acid was added, followed by incubation at room temperature for 15 min. Acidified samples were neutralized with sodium hydroxide. Subsequently, the samples were serially diluted with PBS. TGF-β_1 and -β_2 concentrations were measured by Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems).

Immunohistochemistry

Gliomas of different World Health Organization (WHO) grades of malignancy were analysed on a tissue microarray. The tissue microarray was constructed as previously described (Kononen *et al*., 1998) and contained 14 grade II, 27 grade III and 26 grade IV primary gliomas. Two 0.6 mm cores were punched from each of the 67 tumour paraffin blocks. Tissue microarray sections were incubated according to Ventana automat protocols (Ventana Medical System) using the following rabbit monoclonal primary antibodies: anti-integrin pan-αv (clone EM01309, 1:10 000), anti-integrin αvβ3 (clone EM22703, 1:200), anti-integrin αvβ5 (clone EM09902, 1:1000), anti-integrin αvβ6 (clone EM05201, 1:2000) and anti-integrin αvβ8 (clone EM13309, 1:750) as described (Goodman *et al*., 2012). The immunostaining was performed using anti-rabbit secondary antibodies with iView™ or ultraView™ Amp kits (Ventana). The intensity of integrin staining for pan-αv, αvβ3, αvβ5, αvβ6 and αvβ8 was scored 0 (negative), +1 (weak), +2 (moderate) and +3 (strong) in tumour cells as well as in endothelial cells. Contingency table analysis and Pearson's chi-square tests were used for the analysis of the associations between integrin expression patterns and glioma grade.

Immunofluorescence microscopy

Immunofluorescence studies were carried out on cytospins (5×10^5 cells/slide). Samples were dried for 30 min and fixed in a 4% buffered formalin solution for 10 min at room temperature. Subsequently, antibody to pSmad2 (Cell Signaling) was applied (overnight at 4°C). Binding specificity was controlled by IgG isotype control (Jackson Immunoresearch). For visualization, Cy3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch) was applied. All sections were mounted in Vectashield Mounting Media with 4′,6-diamidino-2-phenylindole (DAPI).

TGF-β activity assay

Transfected mink lung epithelial cells, stably transfected with a luciferase gene driven by TGF-β-responsive plasminogen activator inhibitor-1 promoter sequences (Abe *et al*., 1994), were seeded in 96-well plates and allowed to adhere overnight. Serum-free medium supplemented with recombinant human TGF-β for the generation of a standard curve, naïve glioma-conditioned medium for active TGF-β estimation or heat-treated (80°C, 5 min) conditioned medium for total TGF-β estimation were added to the transfected mink lung epithelial cells, which were harvested 18 h later. Luciferase activity was measured using the luciferase assay system (Promega).

Reporter assay

Intracellular TGF-β signalling was assessed by reporter assays using the pGL3-SBE4-Luc (Zawel *et al*., 1998) reporter plasmid kindly provided by B. Vogelstein (Baltimore, MD). The pGL2-3TP-Luc construct contains a synthetic promoter composed of a TGF-β-responsive plasminogen activator inhibitor-1 promoter fragment inserted downstream of three phorbol ester-responsive elements and was kindly provided by J. Massague (New York, NY). The pGL3-TGF-β_1-453-luc plasmid includes the wild-type TGF-β_1 promoter fragment −453/+1 fused to the luciferase gene (Weigert *et al*., 2002). The pGL3 −1065/+11 plasmid harbours nucleotides −1065 to +11 of the human TGF-β_1 promoter (Weigert *et al*., 2004). Both constructs were generous gifts from C. Weigert (Tuebingen, Germany). Three reporter plasmids with different fragments of the TGF-β_2-promoter were kindly provided by C. Knabbe (Stuttgart, Germany). The pGL3-TGF-β_2-promoter constructs contain the following parts of the promoter: (−1729/+64 wild-type), (−778/+64 wild-type) and (−531/+64 wild-type) (Beisner *et al*., 2006). Dual luciferase/renilla assays were performed with co-transfection of 150 ng of the respective reporter construct and 20 ng of pRL-CMV. Luciferase activity was normalized to constitutive renilla activity (pRL-CMV).

Animal studies

Athymic CD1 nude mice were purchased from Charles River Laboratories. Mice of 6–12 weeks of age were used in all experiments. The experiments were approved by the local authorities. Before all intracranial procedures, mice were anaesthetized by an intraperitoneal 3-component injection comprising fentanyl, midazolam and medetomidin. For intracranial implantation, the mice were placed in a stereotactic fixation device (Stoelting) and a burr hole was drilled in the skull 2-mm lateral and 1-mm posterior to the bregma. The needle of a Hamilton syringe was introduced to a depth of 3 mm. LN-308 glioma cells (10^5) in a volume of 2 µl PBS were injected into the right striatum. Six weeks after tumour inoculation, cilengitide (90 mg/kg) or PBS were injected on five consecutive days. Three hours after the last injection, all mice were sacrificed and brain cryosections were immediately prepared for haematoxylin and eosin staining or for further analyses. pSmad2 expression was assessed by immunofluorescence microscopy as described above. All pictures were taken with a ×63 oil immersion objective (1.4 numerical aperture; Leica Microsystems). A CLSM Leica SP5 microscope (Leica Microsystems) attached to a diode and a helium neon laser were used to provide excitation at 405 nm and 561 nm wavelengths. The emitted fluorescence light was detected via two adjustable photomultiplier detectors. Three single images from different brain sections from one animal were recorded and included in the quantification. The surface detection function from Bitplane Imaris (Bitplane) was used to identify all DAPI-positive areas as nucleus per picture and to exclude extranuclear signals. The total fluorescence intensity of Cy3 in each nucleus was determined and normalized to the nucleus size (fluorescent intensity/μm^2 nucleus area) to avoid false signals because of different nucleus sizes. The total fluorescence intensity of three pictures of three animals per group was averaged.

Statistics

Data are expressed as mean and standard deviation. The experiments shown were repeated three times with similar results. Analysis of significance was performed using the two-tailed Student's *t*-test (Excel, Microsoft) ($*P < 0.05$, $**P < 0.01$).

Results

Integrin expression in human gliomas *in vivo* and in glioma-initiating cell lines *in vitro*

Integrins αvβ3 and αvβ5 are expressed in tumour vasculature and integrins αvβ6 and αvβ8 have been attributed to a role in TGF-β activation (Munger *et al*., 1999; Mu *et al*., 2002; Cambier *et al*., 2005; Alghisi and Ruegg, 2006). This prompted us to determine the expression of these integrins by immunohistochemistry on tissue microarray of gliomas of different WHO grades of malignancy. In glioblastomas, no staining for αvβ6 was found, whereas a rather homogeneous expression pattern was observed for the other αv integrins (Fig. 1A). The integrin expression in endothelial cells versus tumour cells proper was separately analysed in gliomas of different WHO grades. No αvβ6 expression was detected in any glioma either in tumour or endothelial cells. In endothelial cells, the antibodies to pan-αv revealed a homogenous expression pattern in all gliomas. In contrast, the expression of integrin αvβ3 was higher in glioblastoma tissue than in grade II and III tumours, whereas αvβ5 levels were higher in grade II tumours than in grade III and IV gliomas. No staining was observed for αvβ8 in endothelial cells of any glioma (Fig. 1B). Tumour cells within glioblastoma tissue displayed higher αv, αvβ5 and αvβ8 expression levels than in WHO grade II and III tumours. The antibody to integrin αvβ3 revealed faint patterns of expression, but also with the highest levels in grade IV gliomas (Fig. 1C). The expression of integrins αvβ3 and αvβ5 in long-term glioma cell lines has previously been characterized in our laboratory (Maurer *et al*., 2009). Here we confirmed expression of the relevant integrins in LN-308 cells (Fig. 2A) and further demonstrated the presence of these integrins on the surface of glioma-initiating cell lines (Fig. 2B).

Integrin inhibition interferes with Smad2 phosphorylation in human glioblastoma cells

If integrin inhibition impaired TGF-β activation, a reduced phosphorylation of Smad2, a central effector of TGF-β signalling, would be predicted to occur. Accordingly, LN-308 glioma cells treated with antibodies specifically blocking different αv integrins exhibited reduced Smad2 phosphorylation after 48 h (Fig. 3A). Similarly, we used small interfering RNA oligonucleotides to specifically silence the expression of single integrins in LN-308 glioma cells. Compared with control transfectants, the cell surface expression of all integrins was strongly reduced (Supplementary Fig. 1A). Integrin gene silencing was confirmed to be specific, e.g. β5 gene silencing did not affect expression of the other integrins (Supplementary Fig. 1B). Reduced pSmad2 levels were observed relative to cells treated with scrambled control small interfering RNA oligonucleotides (Fig. 3A). Further, Smad2 phosphorylation in LN-308 cells declined as soon as 4 h after exposure to cilengitide, a cyclic RGD pentapeptide with high affinity for integrins αvβ3 and αvβ5 (Mas-Moruno *et al*., 2010) (Fig. 3A). The glioma cell lines LN-18, U87MG, T98G and to a moderate degree LNT-229 too showed reduced pSmad2 levels when treated with cilengitide (Fig. 3A). This reduction in pSmad2 levels was paralleled with an increase in total Smad2/3 levels, most prominently in LNT-229 cells, and probably reflects a negative feedback loop. The loss of pSmad2 induced by cilengitide was already apparent at 1 µM, near the IC_{50} for cilengitide-mediated inhibition of αvβ3/αvβ5-dependent glioma cell attachment (Maurer *et al*., 2009). The addition of exogenous TGF-β_1 at $\geqslant$2 ng/ml restored Smad 2 phosphorylation in LN-308 cells (Supplementary Fig. 2A), but did not restore attachment. Still, we considered the possibility that loss of pSmad2 resulted from detachment alone, and observed that poly-HEMA-mediated detachment promoted a loss of pSmad2 and an increase of total Smad2/3 (Supplementary Fig. 2B). Therefore, to assess whether the loss of pSmad2 is a specific effect of cilengitide or rather a result of detachment, we examined the attached and detached LN-308 cell fractions for pSmad2 in response to cilengitide separately. Interestingly, Smad2 dephosphorylation was even more prominent in those LN-308 cells still

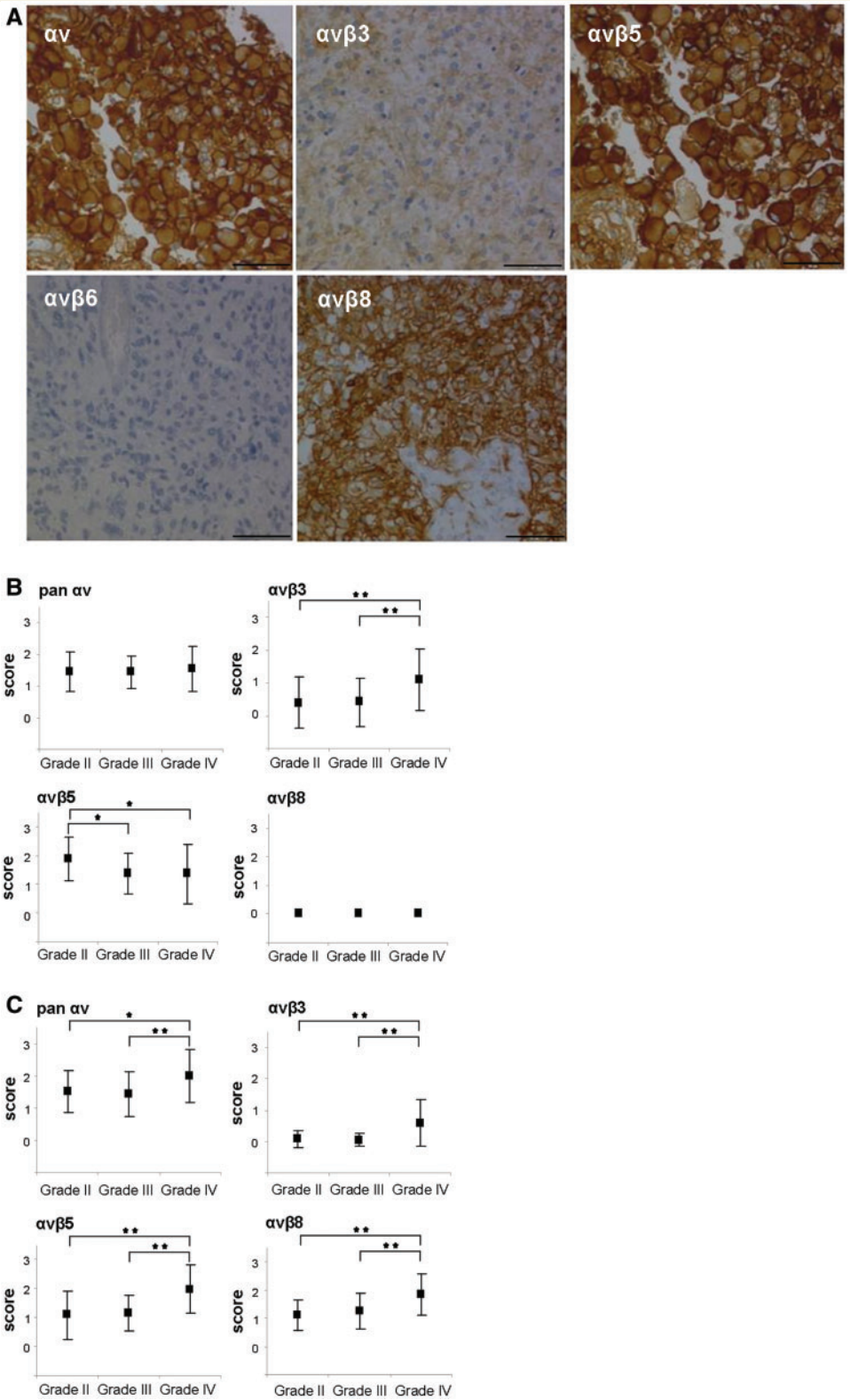

Figure 1 Integrins are expressed in gliomas *in vivo*. (**A**) Paraffin-embedded glioblastoma sections were immunostained with rabbit monoclonal antibodies to integrins pan-αv, αvβ3, αvβ5, αvβ6 and αvβ8. Representative stainings are shown for each integrin. Scale bars = 50 μm. (**B** and **C**) Expression levels for all integrins were quantified separately in endothelial cells (**B**) and tumour cells (**C**). The respective mean scores for WHO grade II, III and IV glioma sections are shown. Significant differences between the three groups are indicated by asterisks ($*P \leqslant 0.05$; $**P \leqslant 0.01$).

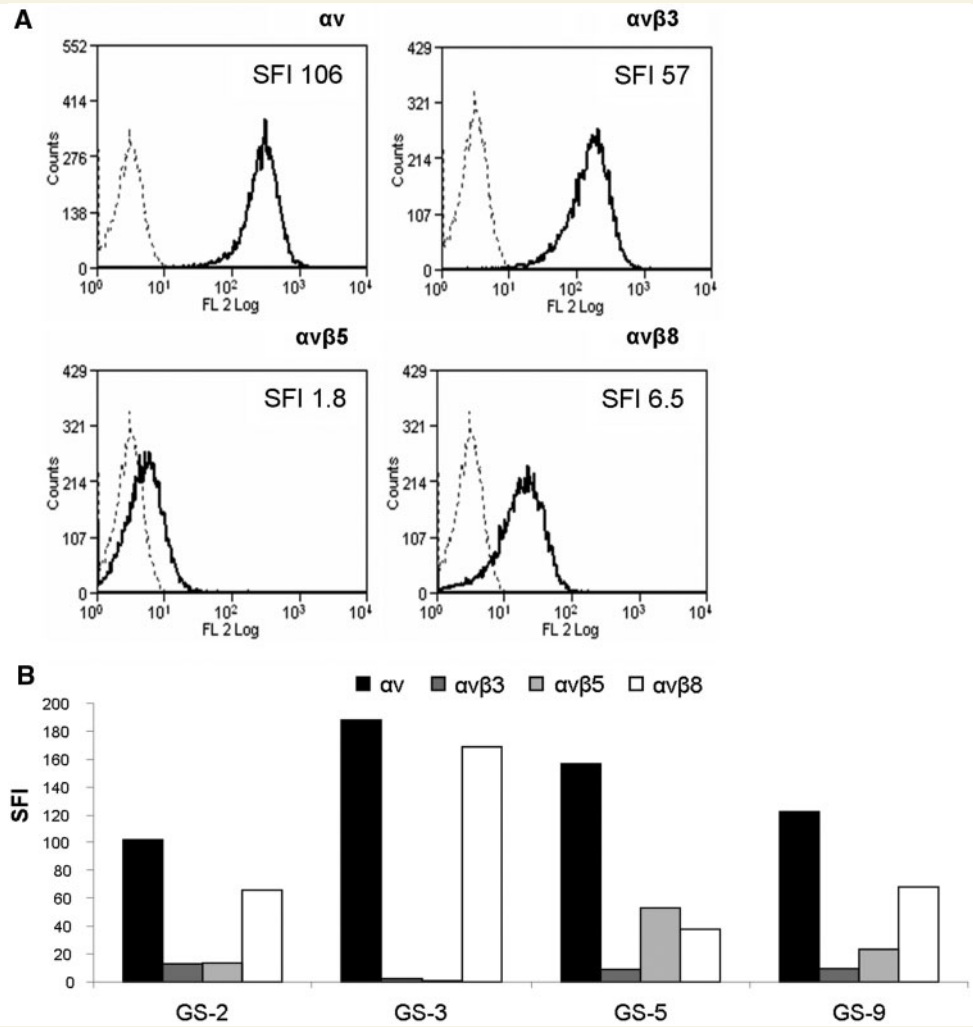

Figure 2 Integrin expression in human glioma cell lines. (**A** and **B**) Integrin expression was determined by flow cytometry of LN-308 (**A**, profiles) or glioma-initiating cell lines (**B**), expressed as specific fluorescence index (SFI) values.

attached to the cell culture flasks than in the detached fraction floating in the media (Supplementary Fig. 2B). Thus interference with Smad2 phosphorylation is likely to be a specific effect of cilengitide on glioma cells and not simply a result of cell detachment.

To confirm the assumption that interference with integrin signalling results in a suppression of the Smad2 pathway, we transiently transfected LN-308 or LNT-229 cells with a reporter construct containing four copies of the Smad-binding site (SBE4-Luc) or the artificial TGF-β reporter plasmid 3TP-Luc. These constructs report TGF-β signalling, which was significantly reduced when the cells were exposed to integrin-blocking antibodies, specific small interfering RNA oligonucleotides or cilengitide, but not with their corresponding controls (Fig. 3B).

Inhibition of integrin activity decreases TGF-β protein release by glioma cells

We next sought to clarify the mechanisms underlying reduced pSmad2 levels and decreased TGF-β-dependent reporter activity and assessed the effects of integrin inhibition on total TGF-β_1 and TGF-β_2 levels in the cell culture media by ELISA. Exposure of LN-308 glioma cells to integrin-blocking antibodies resulted in decreased TGF-β_1 and TGF-β_2 protein levels in the cell culture supernatant after 48 h. The strongest reduction in TGF-β protein levels was observed in cells that had been exposed to the pan integrin αv antibody 17E6, closely followed by αvβ3 and αvβ5 inhibition (Fig. 4A). When using RNA interference to specifically silence single integrins, a strong reduction of TGF-β_1 and TGF-β_2 protein was also observed. Cells with a knock-down of integrin αv displayed the most prominent decrease in TGF-β expression. However, the expression levels of TGF-β were reduced by the knock-down of each integrin chain investigated (Fig. 4B). Additional experiments with a second set of small interfering RNA oligonucleotides resulted in a specific knock-down of integrins (Supplementary Fig. 3A) and a reduction of TGF-β levels (Supplementary Fig. 3B). Finally, exposure of glioma cells to cilengitide also resulted in decreased TGF-β_1 (Fig. 4C), and more prominently TGF-β_2 levels (Fig. 4C) in all glioma cell lines tested. Prominent reductions were noticed in LN-18, T98G and LN-308

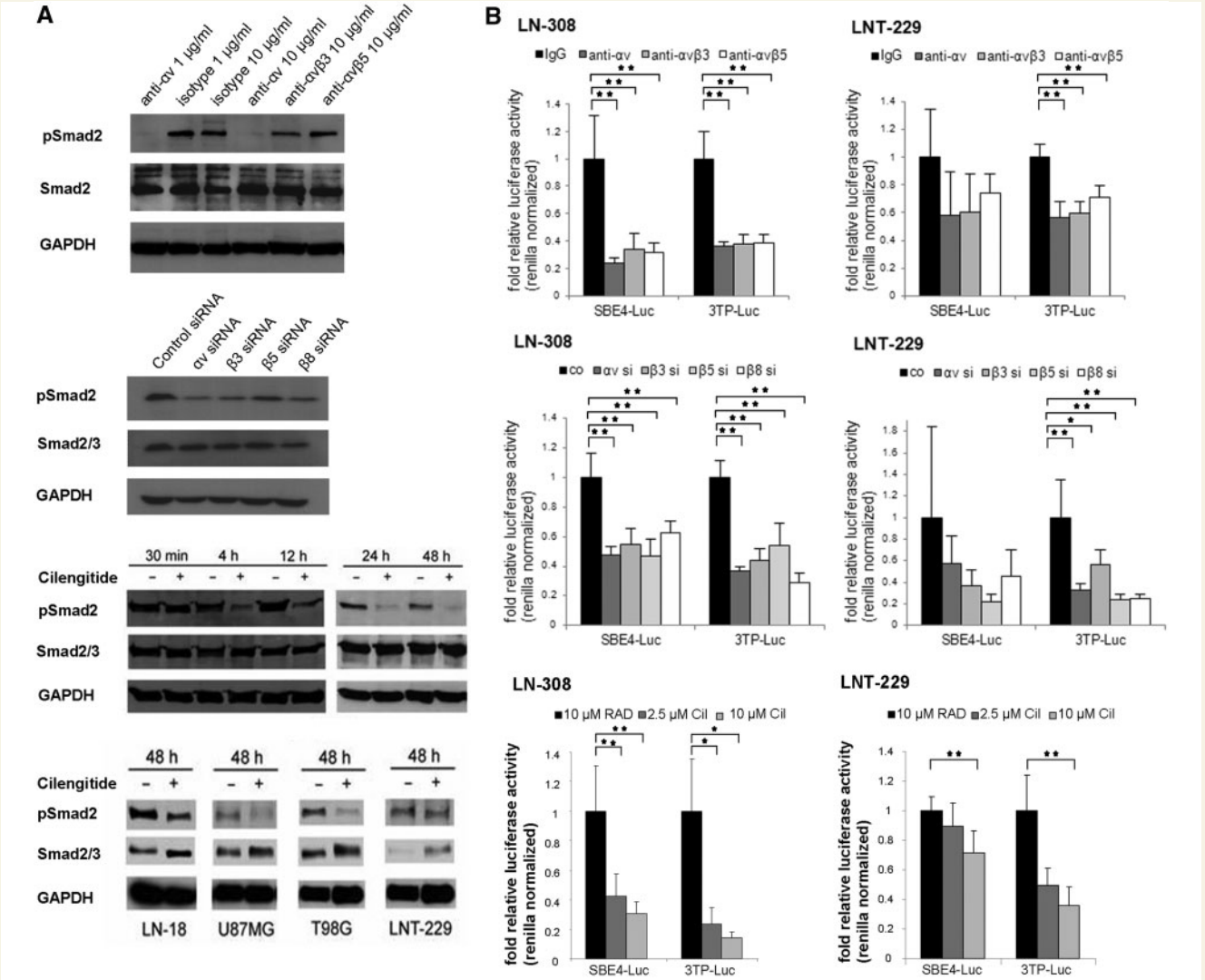

Figure 3 Integrin inhibition decreases Smad2 phosphorylation and TGF-β-induced reporter gene activity. (**A**) Whole cell protein lysates of LN-308 glioma cells, cultured with specific antibodies to different integrins as indicated (*top*), or transiently transfected with small interfering RNA oligonucleotides specific for one integrin or a control small interfering RNA (*middle*), were assessed for pSmad2 and total Smad2/3 levels by immunoblot, using GAPDH as a loading control. In the *bottom* panel, LN-308, LN-18, U87MG, T98G or LNT-229 cells were exposed to control peptide (−) or cilengitide (10 μM) as indicated and assessed for pSmad2 and total Smad2/3 levels. (**B**) LN-308 or LNT-229 cells were exposed to blocking antibodies (*top*) or small interfering RNA oligonucleotides (*middle*) as in (**A**). In the *bottom* panel, LN-308 or LNT-229 cells were exposed to the control peptide RAD (black bars), 2.5 μM (light grey) or 10 μM (dark grey) cilengitide for 24 h. Intracellular TGF-β signalling was assessed by reporter assays using the pGL3-SBE4-Luc and pGl2-3TP-Luc constructs (*$P < 0.05$ and **$P < 0.01$ compared with isotype control and scrambled small interfering RNA or RAD, respectively).

cells below 50% (Fig. 4C). Similarly, most glioma-initiating cell lines responded to cilengitide with decreased TGF-β levels in the cell culture medium (Fig. 4C).

If cilengitide was affecting the TGF-β pathway mainly by acting on the activation of latent TGF-β, then there should be a difference between the ratios of latent as opposed to active TGF-β between control-treated and cilengitide-treated cells. Interestingly, at 1-μM exposure for 24 h, cilengitide had significant effect on active, but not total, levels of TGF-β, whereas both active and total TGF-β were reduced at 10 μM (Fig. 4D), suggesting a dual mode of action of integrin inhibition and TGF-β bioactivity.

Integrin targeting by blocking antibodies, RNA interference or cilengitide suppresses TGF-β expression on the transcriptional level

Based on the observation of reduced TGF-β protein levels in the supernatant, we asked whether integrin inhibition reduces TGF-β expression mainly on the transcriptional level. LN-308 glioma cells treated with antibodies specifically blocking different αv integrins displayed reduced TGF-β_1 and -β_2 messenger RNA expression

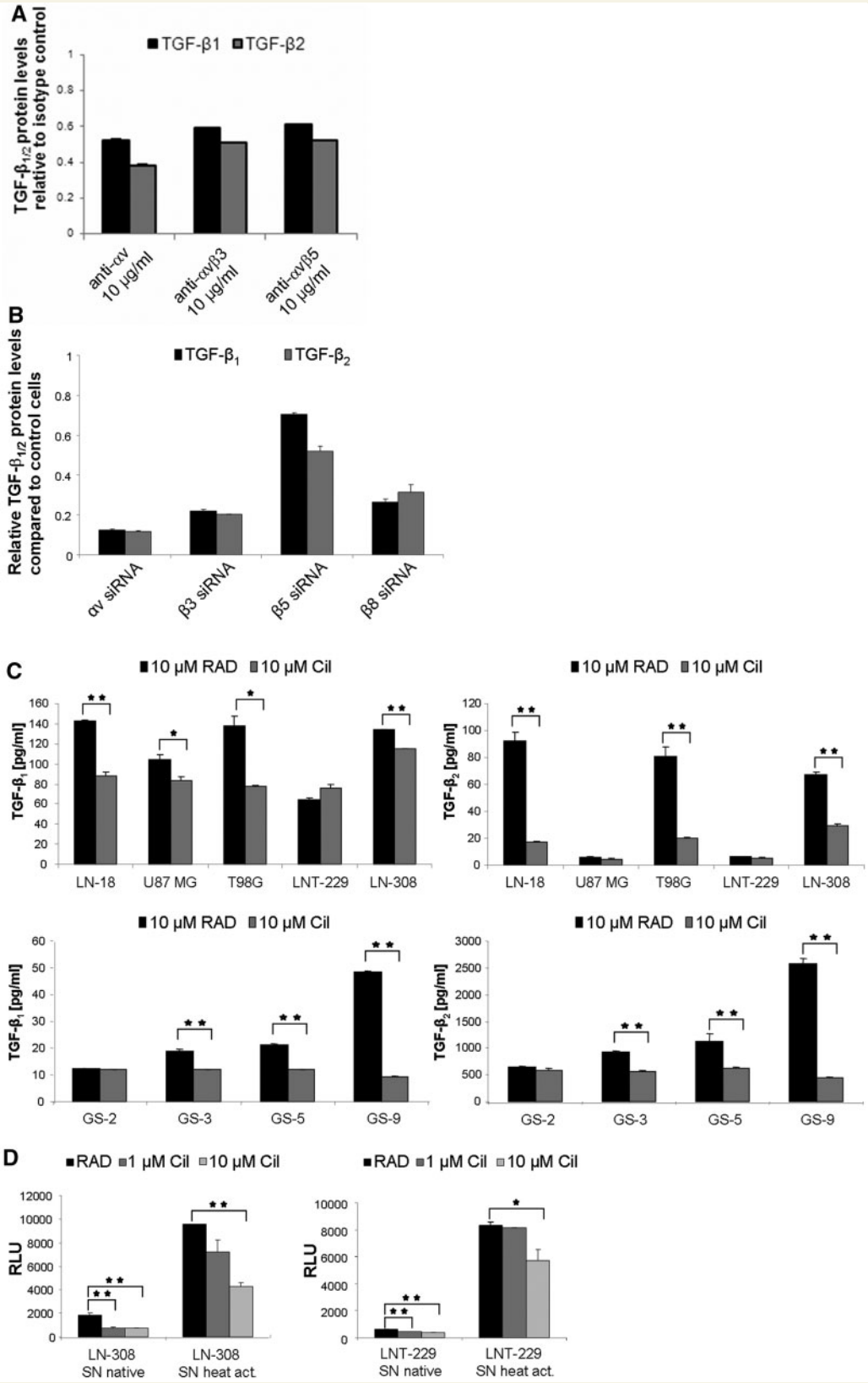

Figure 4 Integrin inhibition suppresses TGF-β protein release. Supernatant from LN-308 glioma cells, exposed to integrin blocking or isotype control antibodies (**A**) or small interfering RNA-mediated integrin gene silencing (**B**), were assessed for TGF-β_1 and -β_2 protein levels by ELISA. (**C**) TGF-β_1 (*left*) or TGF-β_2 (*right*) protein levels in the cell culture supernatant were determined by ELISA after exposure of glioma cells to 10 μM control peptide or cilengitide (Cil) for 48 h. Similar studies were performed using glioma-initiating cell lines (*bottom*). (**D**) LN-308 or LNT-229 cells were exposed to control peptide (RAD) or cilengitide for 48 h as indicated and native or heat-activated supernatants (SN) were assessed in an 18-h transfected mink lung epithelial cells assay for TGF-β bioactivity.

levels as determined by real-time PCR (Fig. 5A). Integrin gene silencing using RNA interference also resulted in decreased TGF-β_1 and TGF-β_2 messenger RNA levels with strongest effects observed after silencing of integrin αv. Finally, we investigated the effects of cilengitide on TGF-β transcripts. Time course analyses of messenger RNA levels in LN-308 cells revealed a reduction in TGF-β_1 and TGF-β_2 messenger RNA (by >50%) within 24 h of exposure to cilengitide, suggesting a transcriptional repression of the TGF-β genes by cilengitide (Fig. 5A). Yet, only minor changes at 4 and 12 h were observed, especially for TGF-β_1, a time point where pSmad levels were already reduced (Fig. 3A).

To confirm the findings obtained by real-time PCR, we examined the effects of all three approaches of integrin inhibition on the TGF-β_1 and TGF-β_2 promoter. Both reporter plasmid constructs are sensitive to changes in the transcription of the respective TGF-β gene. Consistent with the messenger RNA data, targeting integrins with blocking antibodies or small interfering RNA oligonucleotides also induced a reduction of relative luciferase activity in LN-308 and LNT-229 glioma cells (Fig. 5B). A reduction of luciferase activity was also observed when the cells were exposed to cilengitide. Here we examined two constructs of different lengths of the TGF-β_1 promoter and three constructs of different lengths of the TGF-β_2 upstream promoter. For both the TGF-β_1 and -β_2 promoters, a similar reduction in expression levels was noticed independently of the size of the promotor fragment (531–1729 bp upstream).

Cilengitide induces Smad2 dephosphorylation *in vivo*

Compared with blocking antibodies and small interfering RNA oligonucleotides, cilengitide is the clinically most advanced approach to target integrins in glioblastoma. Therefore, we aimed to determine the effect of cilengitide on TGF-β signalling *in vivo*. As a prerequisite, we examined Smad2 phosphorylation in LN-308 glioma cells by immunofluorescence microscopy *in vitro* and observed reduced pSmad2 levels after exposure to cilengitide, using SD-208 as a positive control (Fig. 6A). For *in vivo* analyses, glioma-bearing nude mice were treated with cilengitide or PBS for five consecutive days. Histopathological analyses demonstrated the presence of tumours in all mice. We also confirmed the expression of the target integrins *in vivo*, which was unchanged after 5 days of cilengitide treatment (Fig. 6B). Immunofluorescent stainings displayed heterogeneous effects of cilengitide on Smad2 phosphorylation in the cilengitide-treated mice. In some animals, there was a distinct reduction of pSmad2 levels, whereas other mice appeared not to respond to cilengitide (Fig. 6C). A comprehensive quantification of pSmad2 staining revealed a non-significant trend towards reduced Smad2 phosphorylation in the cohort of cilengitide-treated mice (Fig. 6D).

Discussion

Glioblastoma remains a major challenge in the field of clinical neuro-oncology. The addition of alkylating chemotherapy to radiotherapy has increased the median survival of patients to ~15 months in clinical trials (Stupp *et al*., 2005). Hope for further improvement centres on several novel therapeutic agents that are currently being investigated within clinical trials, notably antiangiogenic agents. The switch of a tumour to an angiogenic phenotype is believed to be one essential step in the multistage process towards malignancy (Hanahan and Folkman, 1996). The tumour vasculature is therefore now seen as a promising therapeutic target in oncology. Antiangiogenic agents in advanced clinical development for glioblastoma include the vascular endothelial growth factor antibody bevazicumab (Vredenburgh *et al*., 2007), the vascular endothelial growth factor receptor antagonist cediranib (Batchelor *et al*., 2010) and the αvβ3 and αvβ5 integrin antagonist, cilengitide (Reardon *et al*., 2008; Stupp *et al*., 2010). Despite advanced clinical evaluation, the mechanisms that mediate clinically beneficial effects of cilengitide in particular, and integrin inhibition in general, remain unknown. Both endothelial and tumour cells might be target cells for such approaches because both express integrins in glioblastoma, albeit in a heterogeneous manner: αvβ3 was expressed typically at high levels in endothelial cells, which were αvβ8-negative, and tumour cells within glioblastomas expressed all three target integrins, but preferentially αvβ5 and αvβ8 (Fig. 1).

TGF-β has been considered as a promising therapeutic target because of its multiple effects contributing to some of the hallmarks of malignant gliomas such as invasiveness or poor immunogenicity (Wick *et al*., 2006). Its regulation by integrins has not been studied in glioblastoma. Because a biologically relevant downregulation of TGF-β should reduce Smad2 phosphorylation, we first explored this pathway in several glioma cell lines. Targeting single integrin complexes with blocking antibodies or small interfering RNA-mediated integrin gene silencing resulted in a downregulation of Smad2 phosphorylation in LN-308 glioma cells (Fig. 3A). Owing to the lack of a commercially available antibody with blocking properties against αvβ8, we could target this integrin only by RNA interference. When LN-308 cells were exposed to cilengitide, a downregulation of Smad2 phosphorylation was observed as soon as 4 h after addition of the compound. This effect peaked at 48 h, and was also seen in other glioma cell lines. As previously reported, some glioma cell lines detach in the presence of cilengitide (Maurer *et al*., 2009), but the effects of cilengitide on Smad2 phosphorylation were independent of detachment. Rather as we show here, they reflect a specific integrin blockade by cilengitide. Smad2 dephosphorylation was more pronounced in cells that remained attached in response to cilengitide compared with detached cells, confirming that Smad2 dephosphorylation is not mediated solely by cell detachment (Supplementary Fig. 2B). Exogenous recombinant TGF-β counteracted the effect of cilengitide on Smad2 phosphorylation, suggesting that cilengitide does not directly block TGF-β receptor-mediated signalling, indeed, as yet few off-target effects have been identified for cilengitide (Supplementary Fig. 2A). Consistent with these findings, the activity of two reporter constructs reflecting Smad2/TGF-β signalling was significantly reduced when the cells were exposed to integrin-blocking antibodies, specific small interfering RNA oligonucleotides, cilengitide, but not with the appropriate controls (Fig. 3B). Next we asked whether the effects of integrin inhibition on pSmad2 levels and

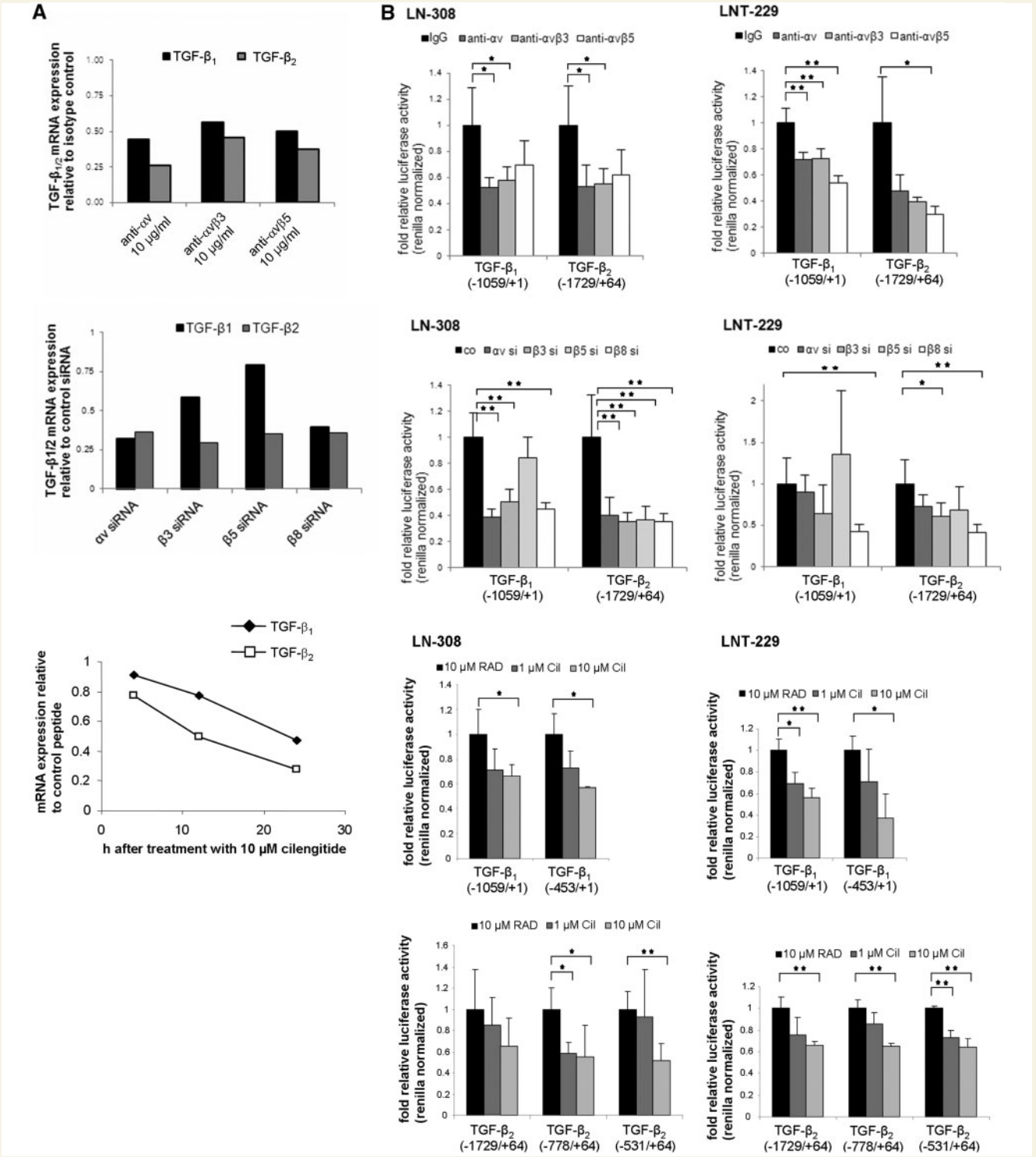

Figure 5 Integrin inhibition suppresses TGF-β gene transcription. (**A**) LN-308 glioma cells were cultured with integrin blocking or isotype control antibodies as indicated. TGF-β_1 and -β_2 messenger RNA expression levels were determined by real-time PCR after 48 h and normalized to isotype-treated cells (*top*). LN-308 glioma cells were transiently transfected with small interfering RNA oligonucleotides specific for one integrin or a control small interfering RNA and TGF-β_1 and -β_2 messenger RNA levels were determined (*middle*). TGF-β_1 or -β_2 messenger RNA expression were assessed by real-time PCR after exposure to 10 μM cilengitide (Cil) at the indicated time points and compared with cells that had been exposed to control peptide (*bottom*). (**B**) Glioma cells were transfected with constructs of different lengths of the TGF-β_1 or TGF-β_2 promoter and treated as in (**A**) (*$P < 0.05$ and **$P < 0.01$ compared with isotype control and scrambled small interfering RNA or the control peptide RAD, respectively).

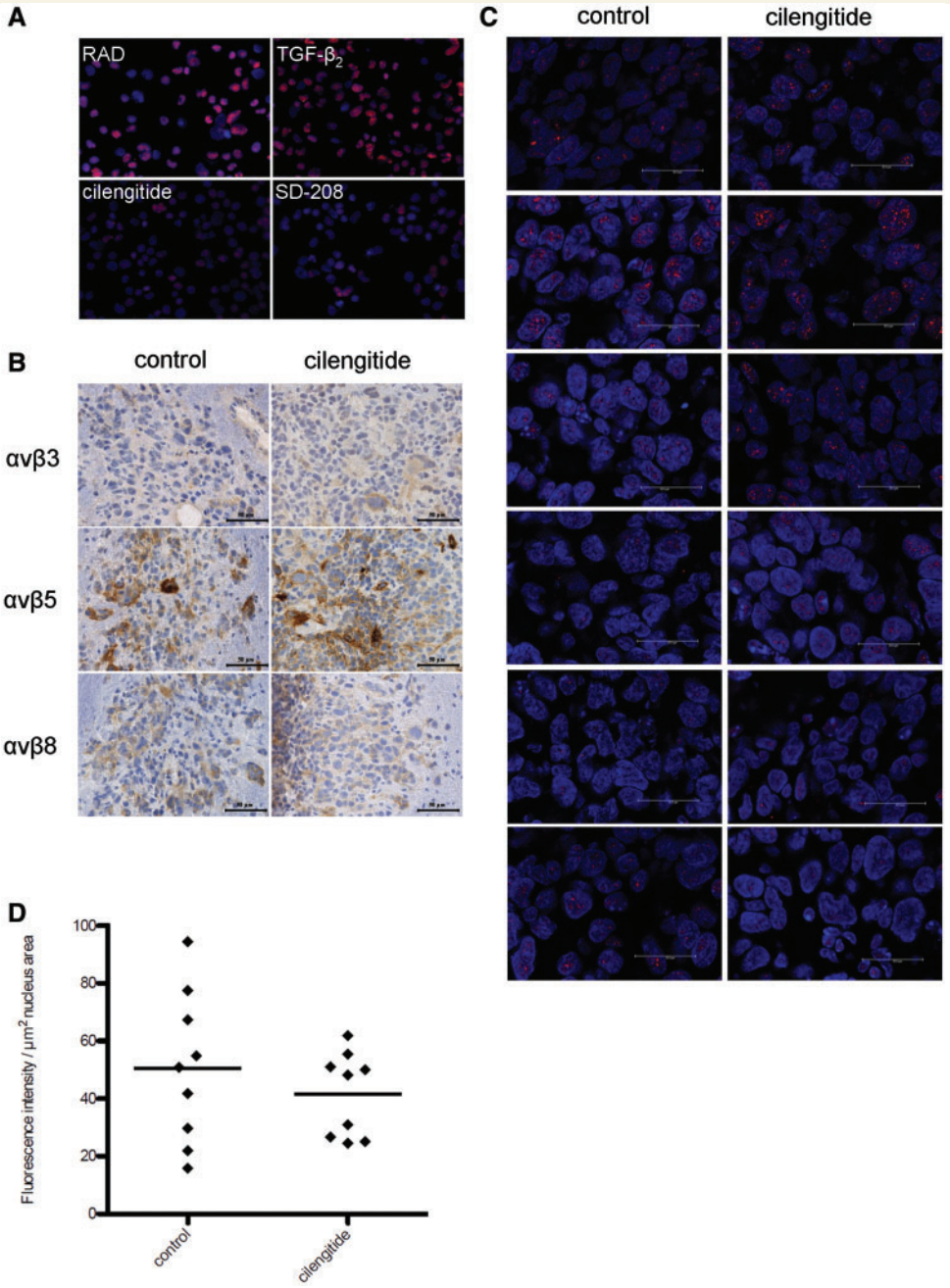

Figure 6 Effects of cilengitide on Smad2 phosphorylation in LN-308 gliomas *in vivo*. (**A**) LN-308 glioma cells were exposed to RAD or cilengitide (10 μM), TGF-β_2 (10 ng/ml) or SD-208 (1 μM) and assessed for pSmad2 expression after 48 h by immunofluorescence microscopy (original magnification ×40). (**B**) LN-308 cells were inoculated intracerebrally in athymic CD1 nude mice. The animals were treated intraperitoneally with cilengitide (90 μg/g body weight) or PBS for 5 days starting 3 weeks after tumour cell inoculation. Subsequently, the animals' brains were removed, shock-frozen and integrin expression was assessed by immunohistochemistry as indicated (original magnification ×10 for haematoxylin and eosin and ×40 for pSmad2). (**C**) pSmad2 levels were assessed by immunohistochemistry (**C**) and quantified (**D**).

TGF-β-dependent reporter activity resulted from a loss of TGF-β protein in the supernatant. Indeed, integrin inhibition with all three approaches reduced TGF-β protein levels in the glioma cell culture supernatant (Fig. 4A–C).

Previous findings suggested a link between integrin signalling and post-transcriptional processing of TGF-β (Munger *et al*., 1999; Mu *et al*., 2002). However, glioma cell supernatants from cilengitide-treated cells showed reduced TGF-β bioavailability, both active and total TGF-β, and not only of active TGF-β, suggesting that interference with activation is not the sole activity of cilengitide. However, Fig. 4D indicates that specifically lower concentrations may exert an effect on TGF-β activity in the absence of effects on total TGF-β.

Indeed, we found that TGF-β_1 and -β_2 messenger RNA levels declined after integrin inhibition with blocking antibodies or RNA interference or exposure of glioma cells to cilengitide (Fig. 5). Additionally, TGF-β gene promoters show reduced activity on integrin inhibition with all three approaches. For both TGF-β_1 and TGF-β_2, similar results were obtained independently from the length of the promoter construct (between 1729–453 bp), indicating that any functionally relevant part of the promoter is even closer to the gene's coding sequence. We also considered the possibility that the cilengitide effects on TGF-β transcription were a result of decreased levels of TGF-β. This interpretation would require that TGF-β controls its own transcription in the experimental system used here. However, previous experiments had not indicated a major contribution of autocrine TGF-β loops to the total TGF-β activity in glioma cell line studies (Leitlein *et al*., 2001; Uhl *et al*., 2004).

The possible clinical importance of our findings was supported by the observation that Smad2 phosphorylation was attenuated in some LN-308 glioma-bearing nude mice treated with cilengitide, identifying the compound as a weak integrin antagonist when pSmad2 phosphorylation is defined as the biological endpoint. The heterogeneous findings here may reflect the clinical situation where cilengitide monotherapy probably neither provides a survival benefit in glioma patients nor causes significant toxicity. A minority of recurrent glioblastoma patients showed durable responses even to cilengitide alone (Reardon *et al*., 2008), but a biomarker to identify such patients has not been established. Absence of target integrin expression would be expected to predict failure of clinical benefit from integrin inhibitors and may in the future be assessed non-invasively by Positron emission tomography (PET) (Schnell *et al*., 2009). In our animal model, there was little response to cilengitide at the level of pSmad2 levels although the target integrins were expressed (Fig. 6), indicating that there are either intracellular mechanisms of resistance operating *in vivo* or that the intrinsic activity of cilengitide is too low, at the dosing schedule used here, or both. However, our findings highlight integrin inhibition as a promising therapeutic approach in glioblastoma and demonstrate that the best way to interfere with integrin signalling still needs to be defined. In summary, the results of this study reveal suppression of the TGF-β pathway as a novel mechanism by which integrin inhibition may be exploited in human patients with TGF-β-dependent tumours such as glioblastoma.

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Conflict of interest

M. Weller received research support from Merck. S. Goodman is an employee of Merck.

Supplementary material

Supplementary material is available at *Brain* online.

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