Expression of the Receptor Tyrosine Kinase Tie2 in Neoplastic Glial Cells Is Associated with Integrin β1-Dependent Adhesion to the Extracellular Matrix

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Abstract

The abnormal function of tyrosine kinase receptors is a hallmark of malignant gliomas. Tie2 receptor tyrosine kinase is a specific endothelial cell receptor whose function is positively regulated by angiopoietin 1 (Ang1). Recently, Tie2 has also been found in the nonvascular compartment of several tumors, including leukemia as well as breast, gastric, and thyroid cancers. There is, however, little information on the function of the Ang1/Tie2 pathway in the non–stromal cells within human tumors. We found that surgical glioblastoma specimens contained a subpopulation of Tie2+/CD31− and Tie2+/GFAP+ cells, suggesting that Tie2 is indeed expressed outside the vascular compartment of gliomas. Furthermore, analysis of a tissue array consisting of 116 human glioma samples showed that Tie2 expression in the neoplastic glial cells was significantly associated with progression from a lower to higher grade. Importantly, Ang1 stimulation of Tie2+ glioma cells resulted in increased adherence of the cells to collagen I and IV, suggesting that Tie2 regulates glioma cell adhesion to the extracellular matrix. Conversely, the down-regulation of Tie2 levels by small interference RNA or the addition of soluble Tie2 abrogated the Ang1-mediated effect on cell adhesion. In studying the expression of cell adhesion molecules, we found that Tie2 activation was related to the up-regulation of integrin β1 levels and the formation of focal adhesions. These results, together with the reported fact that malignant gliomas express high levels of Ang1, suggest the existence of an autocrine loop in malignant gliomas and that a Tie2-dependent pathway modulates cell–to–extracellular matrix adhesion, providing new insights into the highly infiltrative phenotype of human gliomas. (Mol Cancer Res 2006;4(12):915–26)

Introduction

Receptor tyrosine kinases are cell surface proteins that receive signals from extracellular growth factors, which ultimately control biological responses. In normal cells, these receptors exhibit an intrinsic, ligand-controlled tyrosine kinase activity that tightly regulates signal transduction pathways. However, receptor tyrosine kinases become potent oncoproteins when they are mutated, structurally altered, or abnormally expressed. In addition, paracrine and autocrine receptor tyrosine kinase–mediated loops are frequently present in many types of cancers, including gliomas. The characterization of receptor tyrosine kinase function and structures has led to the development of cancer-specific therapies (1).

Tie2 is a receptor tyrosine kinase that was identified as an endothelial cell–specific receptor with a critical role in the modulation of vascular generation and remodeling (2-4). The coordinated expression of Tie2 and its ligands, angiopoietins (Ang1 as an agonist and Ang2 as a context-dependent antagonist), maintains vascular plasticity, and perturbations in this regulation can contribute to abnormal vascular growth (3, 5-7).

Recently, as a result of finding Tie2 expression in nonvascular tissues, several studies were conducted which showed that the functional significance of Tie2 could possibly be broader than originally thought (8, 9). This led to the now well-documented finding that the Ang1/Tie2 signaling pathway is a critical player in preserving the long-term potency of the hematopoietic system (10). Tie2 expression has also been identified in human neural stem cells, and it is maintained during differentiation (11), but not in mature neurons (12). Importantly, Tie2 expression has also been noted in the nonvascular compartment of several tumor types, including leukemia as well as breast, gastric, and thyroid cancers (13-16). In these tissues, Tie2 expression was detected not only in the stromal cells but also in the cells that constitute the origin of the tumor. There is, however, a paucity of information on the function of the Ang1/Tie2 pathway in the nonvascular component of human tumors.

We found for the first time that Tie2 is expressed in the neoplastic glial cells of human glioma specimens. Importantly,

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Tie2 expression was significantly greater in gliomas than in normal human brain tissue, and the extent of increase was associated with the progression from lower grade to higher grade tumors. We further showed that Ang1 treatment phosphorylated Tie2, which regulated the adhesion of glioma cells to components of the extracellular matrix (ECM) through the up-regulation of integrin β1 expression and formation of focal adhesions. Taken together, these observations indicate the existence of Tie2/ligand signaling in glioma cells involved in the cell-to-ECM adhesion properties of human brain tumors.

Results

Tie2 Is Expressed in the Astrocytic Compartment of Human Malignant Gliomas

To initially determine whether Tie2 was expressed in human gliomas, using immunohistochemistry, we analyzed a human tissue microarray composed of 116 glioma specimens (17) of grades II (low grade astrocytoma), III (anaplastic astrocytoma), and IV (glioblastoma multiforme, GBM) of the WHO classification system. We found that Tie2 expression was not restricted to endothelial cells (Fig. 1A). In particular, Tie2 was clearly expressed in the glial cells of 50% of grade III gliomas (23 of 46) and 85% of grade IV gliomas (57 of 67; Table 1). In contrast, Tie2 was not detected in the grade II gliomas ($n = 3$) or normal brain samples ($n = 10$) in the tissue array, except for occasional slightly positive endothelial cells in the grade II gliomas (Fig. 1A; Table 1). Of importance, these results indicated that the expression of Tie2 in extravascular regions of human gliomas is positively associated with increasingly advanced tumor grades ($P < 0.0001$, exact Cochran-Armitage trend test).

To further verify the presence of Tie2 outside the vascular compartment of gliomas, we analyzed Tie2 expression in suspensions of single cells obtained from fresh surgical specimens of human GBMs. As shown in Fig. 1B and Table 2, Tie2 expression was detected, albeit in different percentages of cells, in every glioma sample examined. Fluorescence-activated cell sorting analysis showed, as expected, that CD31+ cells expressed Tie2, identifying the endothelial subpopulation within the tumor. Interestingly, most of the Tie2+ cells were CD31−. To further distinguish the Tie2+ endothelial from the Tie2+ nonendothelial populations, we did fluorescence-activated cell sorting analyses using anti-Tie2, CD31, and CD34 antibodies. Analyses of a surgical GBM specimen showed that 18.61% of the cells were Tie2+, but only 0.57% and 0.27% of the cells coexpressed Tie2/CD31 and Tie2/CD34, respectively. Next, we directly tested whether Tie2 is expressed by astrocytic cells contained in GBM specimens, by using double immunostaining with anti-Tie2 and anti–glial fibrillary acidic protein (GFAP) antibodies. As shown in Fig. 1C, Tie2 expression colocalized with GFAP expression. Altogether, these data suggest that Tie2 is expressed not only in the stromal cells within the GBM tissues, as endothelial cells, but also in a subpopulation of the neoplastic glial cells in malignant gliomas.

We then assessed whether Tie2 is expressed in human glioma cells in a panel of glioma cells. Reverse transcription-PCR (RT-PCR) analysis showed the presence of Tie2 transcripts in U-87 MG and D-54 MG glioma cells, but not in U-251 MG cells (Fig. 2A). PCR products were then sequenced to confirm Tie2 amplification in these two cell lines (data not shown). Consistent with these data, Tie2 protein expression was detected in the membrane fraction of U-87 MG and D-54 MG cells, but not in the membrane fraction of U-251 MG cells, as assessed by Western blotting (Fig. 2B). Differences in the levels of Tie2 expression between glioma tissues and glioma cell lines could be due to a decrease of expression during the *in vitro* culture procedures (18).

Our data showing the expression of Tie2 in the neoplastic astrocytic compartment of malignant gliomas, together with reported studies on the expression of Ang1, the natural agonist ligand of Tie2 (19, 20), suggests the existence of an autocrine loop in glioma cells.

Tie2 Expression Is Associated with Glioma Cell Adhesion to Ang1

To determine the effects of Tie2/Ang1 signaling on glioma cell adhesion, we plated U-87 MG cells (Tie2+ cells), U-251 MG cells (Tie2− cells), and human umbilical vein endothelial cells (HUVEC; positive control) into wells that had been precoated with Ang1. As shown in Fig. 3A and B, U-87 MG cells adhered well to immobilized Ang1, as did HUVEC. In contrast, U-251 MG cells did not adhere to Ang1-precoated wells. To further explore the role of Tie2 in glioma cell adhesion, we generated Tie2-overexpressing U-251 MG cells (U251.Tie2 cells). Using flow cytometry analysis, we confirmed Tie2 expression on the surface of the cells in three different U251.Tie2 clones (Fig. 4A). Then, we evaluated the binding activity of U251.Tie2 cells to Ang1 (Fig. 4B and C). U251.vector cells did not adhere to Ang1-precoated wells, but interestingly, U251.Tie2 clones adhered strongly to Ang1-precoated wells, showing a 10- to 15-fold increase in adhesion compared with U251.vector cells ($P < 0.001$). We then did another adhesion experiment that involved the soluble decoy receptor, Tie2-Fc, in which an extracellular domain of Tie2 was fused in frame with an IgG-Fc fragment, to competitively inhibit Ang1-mediated U251.Tie2 cell adhesion. The treatment with soluble Tie2-Fc inhibited the adhesion of 70% of U251.Tie2 cells to Ang1-precoated wells ($P < 0.001$), whereas human Fc (hFc) had no effect on the adhesion of these cells (Fig. 4D). Counterpart experiments were done in U-87 MG cells plating those cells onto Ang1-coated wells and analyzing their adhesion in the presence of hFc or soluble Tie2-Fc. Results were equivalent to those seen for the U251.Tie2 cells ($P < 0.05$; Fig. 5A).

To confirm the role of Tie2 expression in glioma cell adhesion, we investigated the effect of down-regulating the endogenous Tie2 in U-87 MG cells using small interference RNA (siRNA) and short hairpin RNA (shRNA) targeting two different Tie2 RNA sequences. Tie2 transcript and protein levels significantly decreased after treatment of U-87 MG cells with the silencing RNAs, as assessed by RT-PCR and Western blot analyses (Fig. 5B). Tie2 levels were not down-regulated in cells transfected with their respective controls. Supporting the specificity of the targeting, the expression of epidermal growth factor receptor was not modified after treatment of the cells with the Tie2 silencing RNAs. We then determined whether the adhesion of U-87 MG cells to Ang1 was modified when the Tie2 levels were decreased. As shown in Fig. 5C, U-87 MG

cells transfected with Tie2 siRNA or Tie2 shRNA showed significantly reduced adhesion to Ang1 in comparison with cells transfected with their respective controls ($P < 0.05$).

Tie2 Activation Enhances Glioma Cell Adhesion to ECM by Up-Regulating Integrin β1 Expression

The effect of Tie2 on glioma cell adhesion to the ECM was first studied by analyzing the binding of U251.Tie2 cells to several ECM molecules. This showed that the binding affinity of U251.Tie2 cells to collagen type I and IV, components of the basement membrane, increased by 2.5-fold compared with the adhesion of the Tie2− cells, either parental U-251 MG cells or U251.vector cells (Fig. 6A). However, Tie2 expression did not modify the binding of U251.Tie2 cells to laminin, fibronectin, or vitronectin (Fig. 6A). To determine whether integrins may serve as a mediator of Tie2-mediated cell adhesion, we did adhesion experiments in the presence of the calcium chelator EDTA, a unique inhibitor of integrins. As shown in Fig. 6B, EDTA almost completely inhibited the adhesion of the U251.Tie2 cells to collagens I and IV. That is, EDTA was ~85% effective in blocking the adhesion of these cells to both ECM components. These data suggest that integrins are involved in Tie2-mediated adhesion to ECM molecules.

Next, we screened the expression of several integrins in Tie2-expressing or nonexpressing U-251 MG cells (U-251.Tie2 or parental U-251 MG, respectively) by performing Western blotting and fluorescence-activated cell sorting analyses. We first checked the phosphorylation status of the Tie2 receptor after immunoprecipitation using the anti-Tie2 antibody. As shown in Fig. 6C, total levels of Tie2 were high in the U251.Tie2 cells and the Tie2 receptor was autophosphorylated in these cells, suggesting a constitutively active Tie2 signaling pathway. As expected, vector-transfected U-251 MG cells did not express Tie2. The increase in the total and phospho-Tie2 levels in the U251.Tie2 cells was accompanied by a 2-fold up-regulation of the integrin β1 protein, compared with the levels in U-251.vector cells. However, there were no differences within this isogenic system in levels of expression of integrin β4, as assessed by Western blotting (Fig. 6C), or of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, as assessed by flow cytometry (data not shown). In addition, the levels of total and phosphorylated Tie2, as well as the differential expression pattern of integrin molecules, were similar in the three U251.Tie2 clones studied

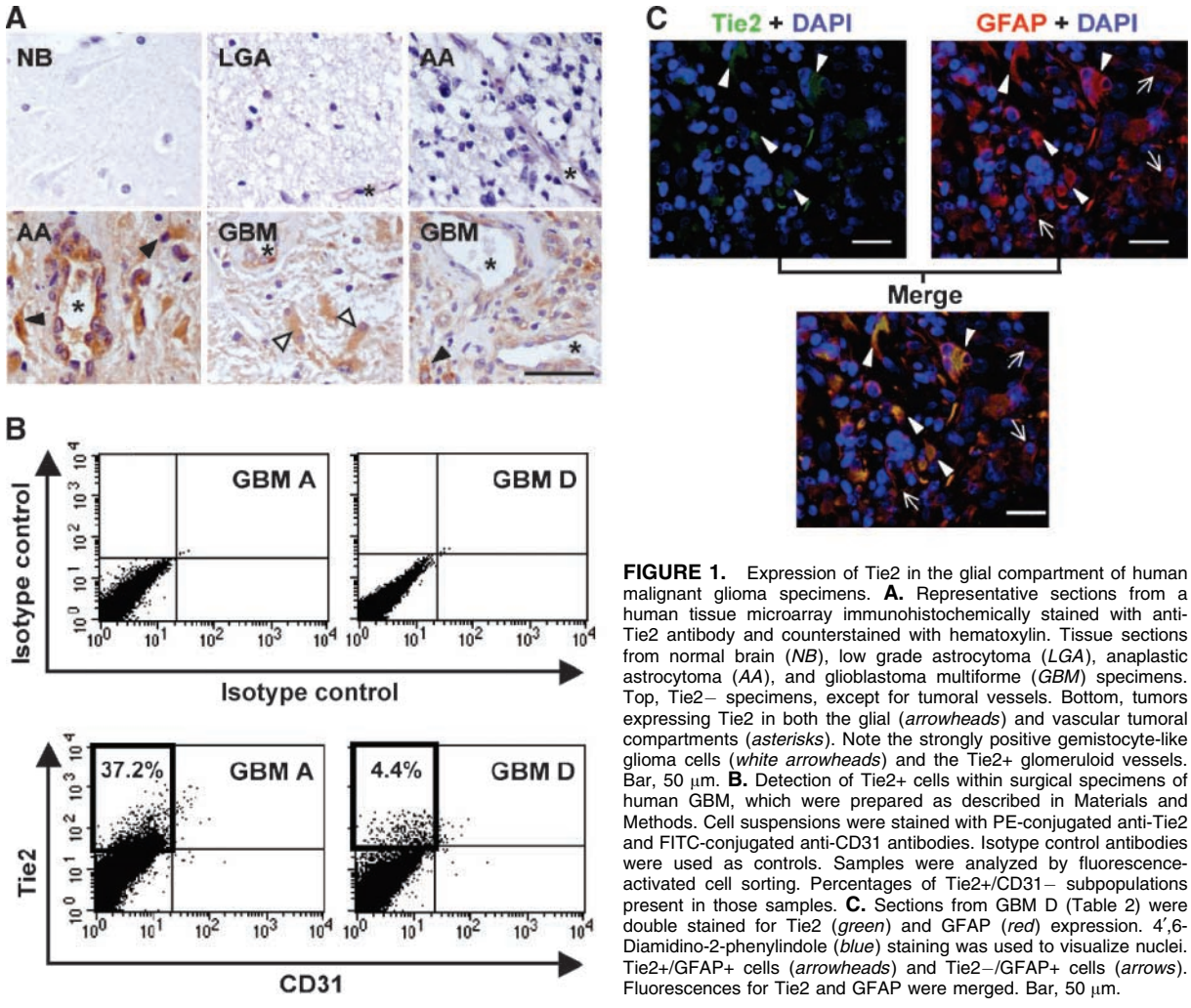

FIGURE 1. Expression of Tie2 in the glial compartment of human malignant glioma specimens. **A.** Representative sections from a human tissue microarray immunohistochemically stained with anti-Tie2 antibody and counterstained with hematoxylin. Tissue sections from normal brain (*NB*), low grade astrocytoma (*LGA*), anaplastic astrocytoma (*AA*), and glioblastoma multiforme (*GBM*) specimens. Top, Tie2− specimens, except for tumoral vessels. Bottom, tumors expressing Tie2 in both the glial (*arrowheads*) and vascular tumoral compartments (*asterisks*). Note the strongly positive gemistocyte-like glioma cells (*white arrowheads*) and the Tie2+ glomeruloid vessels. Bar, 50 μm. **B.** Detection of Tie2+ cells within surgical specimens of human GBM, which were prepared as described in Materials and Methods. Cell suspensions were stained with PE-conjugated anti-Tie2 and FITC-conjugated anti-CD31 antibodies. Isotype control antibodies were used as controls. Samples were analyzed by fluorescence-activated cell sorting. Percentages of Tie2+/CD31− subpopulations present in those samples. **C.** Sections from GBM D (Table 2) were double stained for Tie2 (*green*) and GFAP (*red*) expression. 4′,6-Diamidino-2-phenylindole (*blue*) staining was used to visualize nuclei. Tie2+/GFAP+ cells (*arrowheads*) and Tie2−/GFAP+ cells (*arrows*). Fluorescences for Tie2 and GFAP were merged. Bar, 50 μm.

Table 1. Summary of the Tissue Array Samples by the Presence or Absence of Tie2 Expression in Glioma Cells and Their Different Degrees of Malignancy

Tumor type	Total (*n*)	Tie2+, *n* (%)
GBM (WHO grade IV)	67	57 (85)
Anaplastic astrocytoma (WHO grade III)	46	23 (50)
Low grade astrocytoma (WHO grade II)	3	0 (0)

NOTE: $P < 0.0001$, determined using an exact Cochran-Armitage trend set.

(data not shown). To ascertain the role of integrin β1 in Tie2-mediated cell adhesion, we included a function-blocking anti-β1 integrin antibody in the adhesion assays. This caused the adhesion of U251.Tie2 cells to collagen I to be completely blocked (Fig. 6D).

Because integrin activity results in the phosphorylation and activation of the cytoplasmic tyrosine kinase focal adhesion kinase (FAK; ref. 21), we then checked the phosphorylation status of FAK after Tie2 activation. Although Tie2 transfer into U-251 MG cells did not modify the total levels of FAK, the levels of phosphorylated FAK were increased compared with those in U251.vector cells (Fig. 6C). Tie2/integrin/ECM-dependent adhesion was further shown by the recruitment of paxillin into focal adhesions. Thus, as shown in Fig. 6E, U251.Tie2 cells incubated on collagen I–coated slides exhibited an increase in the antipaxillin immunostaining of focal adhesions, which characteristically appear as elongated dashes or short streaks. In control U251.vector cells, paxillin-immunoreactive focal adhesions were relatively sparse and poorly defined. Taken together, these data suggest that integrins play an important role in the adhesion of U-251 MG cells modified to express the Tie2 receptor.

A second set of experiments was then done to confirm the role of endogenous Tie2 in modulating integrin expression and glioma cell adhesion. We treated U-87 MG cells with the ligand Ang1 to activate the Tie2-dependent pathway. Using immunoprecipitation and antiphosphotyrosine antibodies, we detected phosphorylated Tie2 after Ang1 treatment (Fig. 7A). Then, we analyzed integrin β1 expression in U-87 MG cells after incubation with Ang1 for the time indicated. As shown in Fig. 7B, Ang1 up-regulated the integrin β1 levels 2-fold in U-87 MG cells within 3 h after Ang1 stimulation, and the levels were maintained for at least 12 h. Integrin β4 was not detected in U-87 MG cells in the absence or presence of Ang1 (data not shown). In accordance with Western blot data, flow cytometric analyses showed that Ang1 increased surface levels of integrin β1 in U-87 MG cells (Fig. 7C). To ascertain the role of the Tie2 receptor in the Ang1-mediated increase in integrin β1 expression, we transfected U-87 MG cells with Tie2-targeted siRNA and then incubated the cells with Ang1. Tie2 siRNA, but not control siRNA, inhibited the Ang1-induced expression of integrin β1 in U-87 MG cells. Specifically, the down-regulation of Tie2 levels was accompanied by an ~50% decrease in integrin β1 protein levels compared with cells transfected with control siRNA (Fig. 7D).

Next, we tested whether Ang1/Tie2 signaling was related to glioma cell adhesion to ECM components. This showed that Ang1 increased the attachment of Tie2-expressing U-87 MG cells to collagen I and IV ($P < 0.005$ and $P < 0.05$, respectively). However, U-87 MG cells transfected with Tie2 siRNA did not exhibit Ang1-induced cell adhesion to any of the ECM components compared with cells transfected with control siRNA (Fig. 7E). Taken together, these data suggest that Ang1 signals through Tie2 modulate the activity of the adhesion receptor integrin β1 on the surface of glioma cells to ECM components.

Discussion

Our data showed that the tyrosine kinase receptor Tie2 is expressed not only in the vascular compartment of gliomas but also in the neoplastic astrocytic cells, with the degree of expression significantly associated with progression to malignancy. The results presented here, together with previous reports on the expression of Ang1 in GBMs (19, 20), easily suggested the existence of an autocrine ligand/receptor signaling loop in these tumors. In fact, the expression of Ang1 in gliomas has been reported to be associated with malignancy, and to be present in most of the neoplastic astrocytic cells within GBMs (19, 20). Thus, the activation of Tie2 by phosphorylation resulted in the up-regulation of integrin β1, the phosphorylation of FAK, the formation of focal adhesions, and the tight adhesion of glioma cells to components of the ECM (collagens I and IV) that are mainly present in the basement membrane.

Although Tie2 was first identified as an endothelial cell–specific receptor (2, 3), recent research has shown the existence of this angiopoietin receptor in the non–stromal cell population of cancer tissues. In particular, Tie2 expression has been found in the cells of such cancers as leukemia and gastric, thyroid, and breast cancers (13-16). Our results provide the first evidence that Tie2 is expressed in the neoplastic astrocytic cells of brain tumors. In fact, only 0.8 ± 0.4% of cells obtained from the surgical tumors were CD31+Tie2+, which is consistent with the recent findings of De Palma et al. (22). In that report, the recruitment of Tie2+ cells from the host into tumors was analyzed in transgenic mice expressing GFP (Tie2-GFP) regulated by transcription regulatory sequences of the Tie2 gene. When N202 mouse mammary tumors were grown s.c. in the Tie2-GFP mice, only 1% to 2% of total cells obtained from the tumors were GFP+, and these consisted mainly (~60% of the Tie2+ cell population) of endothelial cells (22). However, in fresh surgical glioblastoma specimens, we observed that Tie2 was expressed in a higher percentage of cells ranging from 6% to 38%, with most of Tie2+cells being

Table 2. Summary of Flow Cytometric Analysis of Tie2 Expression in GBMs

GBM sample	Tie2+ (%)	Tie2+CD31− (%)	Tie2+CD31+ (%)
A	37.64	37.26	0.38
B	37.71	Not determined	Not determined
C	11.65	10.66	0.99
D	5.62	4.40	1.22

NOTE: Tie2+ cells were present, although to different extents, in both nonendothelial cells (Tie2+/CD31−) and endothelial cells (Tie2+/CD31+) in every sample examined.

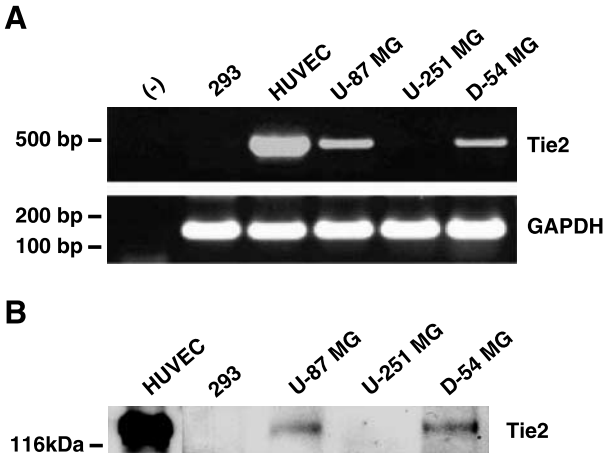

FIGURE 2. Tie2 is expressed in human glioma cell lines. **A.** The presence of Tie2 transcripts is shown in a panel of human glioma cell lines, as assessed by RT-PCR. HUVECs and 293 cells were used as positive and negative controls, respectively. Amplification of glyceraldehyde-3-phosphate dehydrogenase is shown as an internal control. (–), PCR reaction without RNA template. **B.** Immunoblot analyses of Tie2 protein expression in glioma cultures. Tie2 is detected in the membrane fraction of U-87 MG and D54 MG glioma cells but not in U-251 MG and 293 (negative control) cells. HUVEC lysates were used as a positive control.

CD31−. Although the possibility of a population of monocytes or pericyte precursors Tie2+CD31− may not be completely excluded, those populations should account for <1% of the tumoral cells (22). Therefore, collectively, data from the tissue array, colocalization of Tie2 expression with GFAP, a marker of glial cells, and the existence of Tie2 in glioma cell lines, indicated the expression of Tie2 in the neoplastic glial cells of malignant gliomas. Interestingly, the expression of Tie2 in glioma cells seems to be clinically relevant because it was significantly related to malignant progression. In accordance with our data, Tie2 was identified, in a gene expression profiling of brain tumor tissues from 25 patients, as a gene that robustly distinguished GBM tumors from other types of gliomas (23).

Previous studies have shown that Ang1 is involved in endothelial (4, 7) or hematopoietic cell adhesion to components of the ECM and stromal cells by stimulating the Tie2-dependent pathway (10, 24) or by directly binding to integrins (25, 26). In this study, we showed that adhesion of glioma cells to Ang1 and components of the ECM depends on Tie2 expression. Thus, we observed that Tie2 transfer into Tie2− cells U-251 MG resulted in the dramatic adhesion of those cells to the ligand and to collagens I and IV. Conversely, the down-regulation of Tie2 expression in U-87 MG cells inhibited cell adhesion to Ang1 and the ECM. Several studies have been reported that support the regulatory roles of Ang1 as an adhesion protein (25, 27) that, at least in some settings, is related to cell survival, as is the case of the adhesion of skeletal and cardiac myocytes to Ang1, conferring cardioprotection (26). Similarly, Ang1 promotes the adhesion of Tie2+ hematopoietic stem cells to fibronectin, collagen, and osteoblasts (10, 24), which protects the hematopoietic stem cells from myelosuppressive stress.

Interactions between tumor cells and the ECM strongly influence tumor development, affecting cell proliferation and survival, as well as the ability of the tumor cells to migrate beyond the original location of the tumor. In this regard, our data showed that stable Tie2 activation in human glioma cells promoted their adhesion to collagens I and IV by up-regulating integrin β1, a common denominator among the α-β heterodimers that can bind to the ECM proteins present in the perivascular space (i.e., collagen, fibronectin, and laminin). Recently, Cascone et al. showed that Tie2 associates constitutively with integrin $\alpha_5\beta_1$ in endothelial cells, thereby regulating the engagement to fibronectin and the sensitization of the receptor to low Ang1 concentrations (28). Altered expression of integrins has been detected in the majority of malignant tumors and seems to be one of the abnormalities that promotes tumor progression (29). For example, among the gliomas, integrin β1 is overexpressed in most of the GBMs (30, 31), with abundant evidence supporting the importance of integrin β1 in the malignant phenotype of gliomas (32). Thus, the induction of integrin β1 overexpression in C6 glioma cells that were implanted intracranially into the brains of nude mice led to diffuse invasion in the brain (30).

Integrins are also known to cooperate with growth factors to regulate several cellular functions. Indeed, many cellular

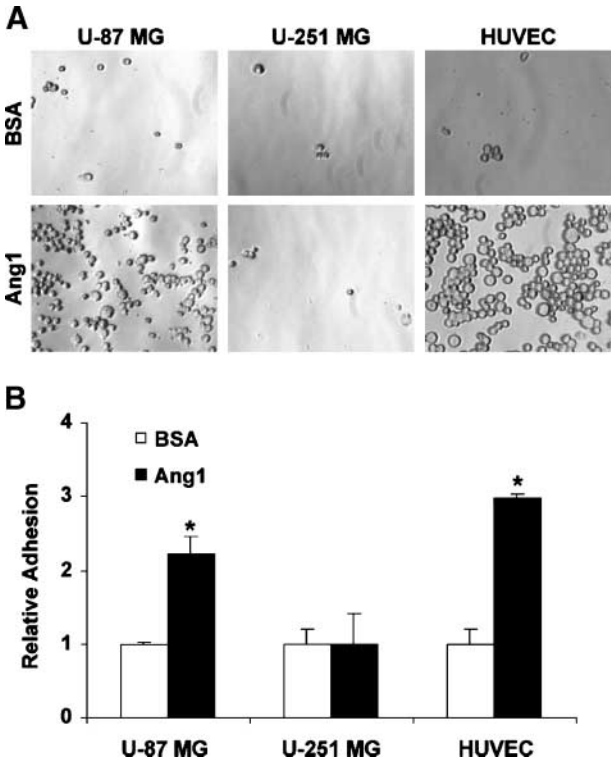

FIGURE 3. Adhesion of glioma cells to Ang1. **A.** U-87 MG or U-251 MG cells (5×10^4 cells) were plated on wells coated with 10 μg/mL of Ang1 or control BSA for 1 h. HUVECs were used as a positive control for this experiment. Results from a representative experiment testing the adherence of glioma cells to BSA- or Ang1-coated wells and fixed with paraformaldehyde (magnification, ×100). **B.** The fold increase in glioma and HUVEC cell adhesion to Ang1 is shown. Cells were stained with crystal violet and absorbances were measured (570 nm). Results are expressed as the fold increase in the adhesion of cells to Ang1 compared with BSA, equal to 1. Columns, means from at least three independent experiments, each done in triplicate; bars, SD; *, $P < 0.001$, compared with adhesion to BSA-coated wells.

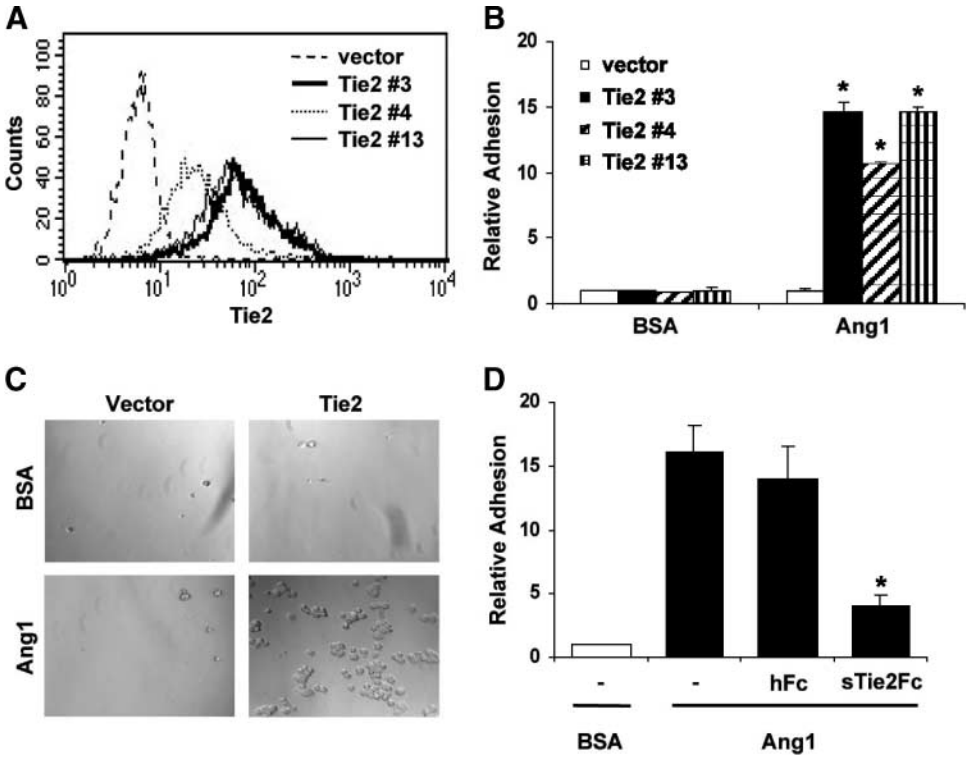

FIGURE 4. Tie2 expression modulates glioma cell adhesion to Ang1. **A.** Characterization of the Tie2-expressing U251.Tie2 cells. Fluorescence-activated cell sorting analysis of the expression of Tie2 in the surface of U251.vector and the U251.Tie2 stable clones using anti-Tie2 antibody. Fluorescence intensity is expressed in arbitrary units. **B.** Adhesion of U-251 MG cells to Ang1. Glioma cells (1×10^5) were plated onto BSA- or Ang1-coated wells. Results are expressed as the fold increase in the adhesion of cells to Ang1 compared with BSA, equal to 1. Experiments were done in triplicate. *, $P < 0.001$ compared with adhesion to BSA-coated wells. **C.** Representative experiment of U251.vector and U251.Tie2 (clone no. 3) cell adhesion to BSA- or Ang1-coated wells (magnification, ×100). **D.** Blockage of U251.Tie2 cell adhesion to Ang1 by soluble Tie2-Fc (sTie2Fc). Adhesion of U251.Tie2 cells (clone no. 3, 1×10^5) to coated Ang1 (10 μg/mL) was measured in the absence or presence of 50 μg/mL hFc or sTie2-Fc. Results are expressed as the fold increase in the adhesion of cells to Ang1 compared with BSA, equal to 1. Columns, means from at least three independent experiments, each done in triplicate; bars, SD; *, $P < 0.001$, compared with hFc-treated U251.Tie2 cells.

responses to soluble growth factors, such as epidermal growth factor and platelet-derived growth factor, depend on the cell adhering to a substrate via integrins. That is the essence of anchorage-dependent cell survival and proliferation, and integrins lie at the basis of these phenomena (33). A study of a panel of glioblastomas indeed showed that integrin β1 expression was enriched in growth factor–overexpressing areas, such as areas expressing epidermal growth factor receptor and platelet-derived growth factor receptor-α (31). By modulating the adhesive properties of glioma cells, and in particular, by increasing integrin β1 expression, and by activating integrin β1 signaling, Ang1/Tie2 might contribute to the increased aggressive behavior of human brain tumors.

In conclusion, using several approaches, we have shown the expression of Tie2 not only in the human tumoral vascular system, but also in human glioma cells. These results, together with the fact that malignant gliomas express high levels of Ang1, suggest that a previously unknown Ang1/Tie2 pathway in glioma cells controls critical aspects of the neoplastic behavior of gliomas. This Tie2-dependent pathway modulates cell-to-ECM adhesion by up-regulating integrin β1 levels. This provides new insight into the highly infiltrative phenotype of human gliomas. Given the diverse functions of integrins and ECM proteins in glioma tumorigenesis, the Ang1/Tie2/integrin β1–ECM interaction constitutes a logical area for further research as well as a potential target for therapeutic strategies directed against glioma cells and their microenvironment.

Materials and Methods

Glioma Tissue Microarray and Immunohistochemical Analysis

A human glioma tissue array was constructed as described previously (17). Briefly, glioma samples from patients were fixed, embedded in paraffin, sectioned, and stained with H&E, and reviewed by a neuropathologist for representative tumor regions. Glioma samples were then grouped according to the diagnostic criteria of the WHO 2000 classification system. A tissue microarray was constructed from the selected gliomas with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). The tissue microarray included representation in a slide of 10 specimens of normal brain, 3 specimens of grade II glioma, 46 specimens of grade III glioma, and 67 specimens of grade IV glioma. Duplicate samples of each tumor were obtained from representative areas of either one or two donor blocks using a 0.6 mm punch.

Paraffin-embedded sections of gliomas in an array were incubated with two different anti-Tie2 antibodies targeting

different protein domains [C-20, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); and AF313, 1:10 (R&D Systems, Minneapolis, MN)]. Slides were then processed as described previously (34). Sections were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO). Negative controls consisted of sections exposed to secondary antibodies in the absence of incubation with corresponding primary antibodies. Moreover, negative controls consisted of normal brain specimens (n = 10) included in the same slide with the tumor samples. Samples were analyzed by Dr. Fuller (Department of Neuropathology, M.D. Anderson Cancer Center), and then by two independent researchers, who categorized the samples as showing either positive or negative Tie2 immunoreactivity outside the vascular structures.

Preparation of Single Cell Suspensions from Surgical Specimens of Human GBM

GBM specimens for the preparation of single cell suspensions were obtained postsurgery. Tumors were dissociated enzymatically, as described previously (35, 36). Briefly, minced tumors were placed into artificial cerebrospinal fluid that contained 124 mmol/L of NaCl, 5 mmol/L of KCl, 1.3 mmol/L of $MgCl_2$, 2 mmol/L of $CaCl_2$, 26 mmol/L of $NaHCO_3$, and 10 mmol/L of D-glucose (pH 7.35). After 15 min, the tumors were treated with 0.1% collagenase and 0.1% hyaluronidase (both from Sigma-Aldrich) in low-Ca^{2+} artificial cerebrospinal fluid (124 mmol/L NaCl, 5 mmol/L KCl, 3.2 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$, 26 mmol/L $NaHCO_3$, and 10 mmol/L D-glucose) for 1 h at 37°C. Dissociated cells were further treated with 0.05% trypsin (Life Technologies, Rockville, MD) in low-Ca^{2+} artificial cerebrospinal fluid for 10 min to obtain a single cell suspension for flow cytometric analysis.

Cell Culture

Human glioma cell lines—U-87 MG (early passages of cells), U-251 MG (both from the American Type Culture Collection, Manassas, VA), D-54 MG (a generous gift from Dr. Bigner, Duke University, Durham, NC), and 293 cells (Microbix Biosystem, Inc., Toronto, ON, Canada)—were maintained in DMEM/F-12 (1:1, vol/vol) supplemented with 10% fetal bovine serum. HUVECs (from passages 2 to 7) were maintained as recommended by the provider (Clonetics, San Diego, CA).

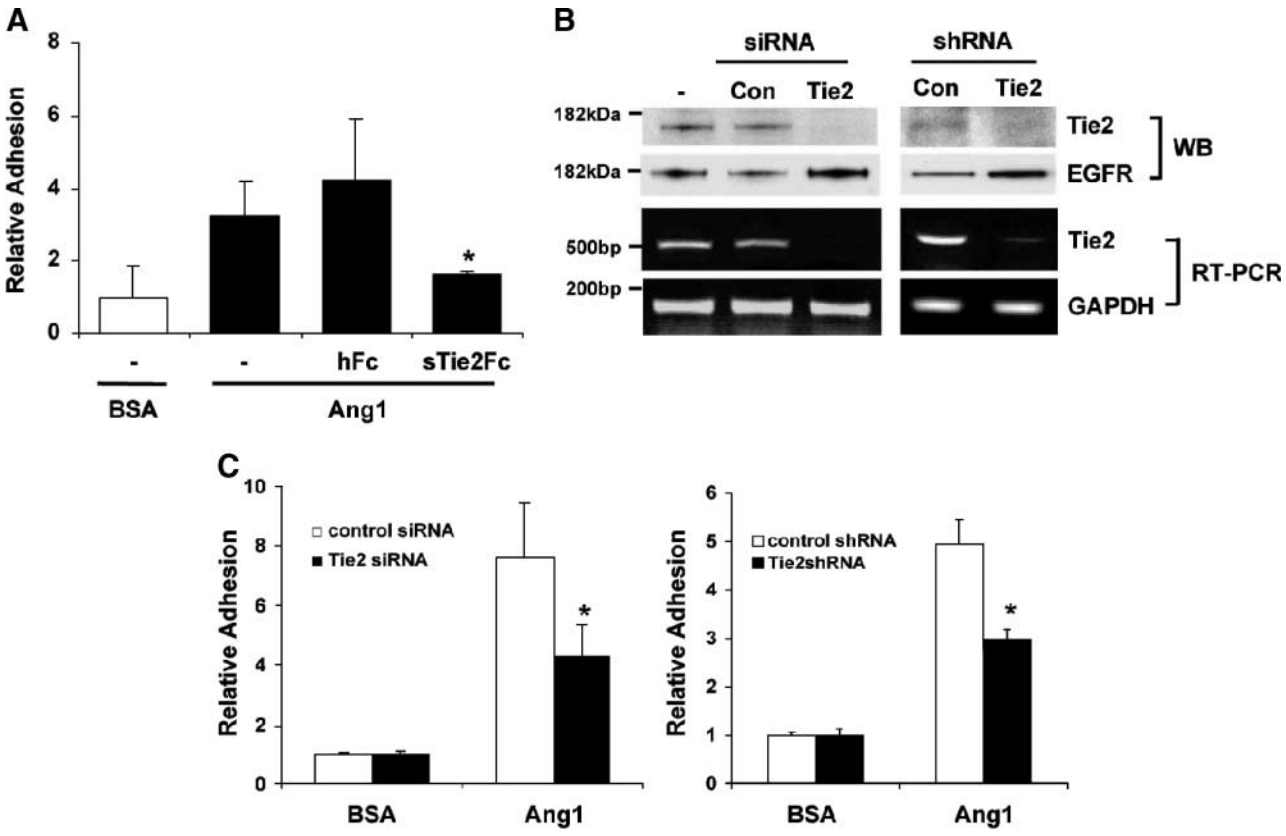

FIGURE 5. Treatment with sTie2-Fc or Tie2 siRNA decreases adhesion of Tie2+ glioma cells, U-87 MG, to Ang1. **A.** Inhibition of U-87 MG cell adhesion to Ang1 by sTie2-Fc. Adhesion of U-87 MG cells (1×10^5) to Ang1 was measured in the absence or presence of 50 μg/mL of hFc or sTie2-Fc. Results are expressed as the fold increase in U-87 MG cell adhesion to Ang1 in the presence of sTie2Fc compared with hFc, equal to 1. Experiments were done in triplicate. *, $P < 0.05$ compared with hFc-treated U-87 MG cells. **B.** RT-PCR and Western blotting analyses showed down-regulation of Tie2 mRNA and protein levels, respectively, in U-87 MG cells after transfection with Tie2 siRNA, or Tie2 shRNA compared with controls. Glyceraldehyde-3-phosphate dehydrogenase and epidermal growth factor receptor levels are shown as the loading controls. **C.** Adhesion to Ang1 of U-87 MG cells (1×10^5) transfected with controls, Tie2 siRNA (50 nmol/L), or Tie2 shRNA and then plated onto 10 μg/mL of BSA- or Ang1-coated wells. Results are expressed as the fold increase in the adhesion of cells to Ang1 compared with BSA, equal to 1. *, $P < 0.05$ compared with control siRNA- or shRNA-transfected U-87 MG cells in Ang1-coated wells.

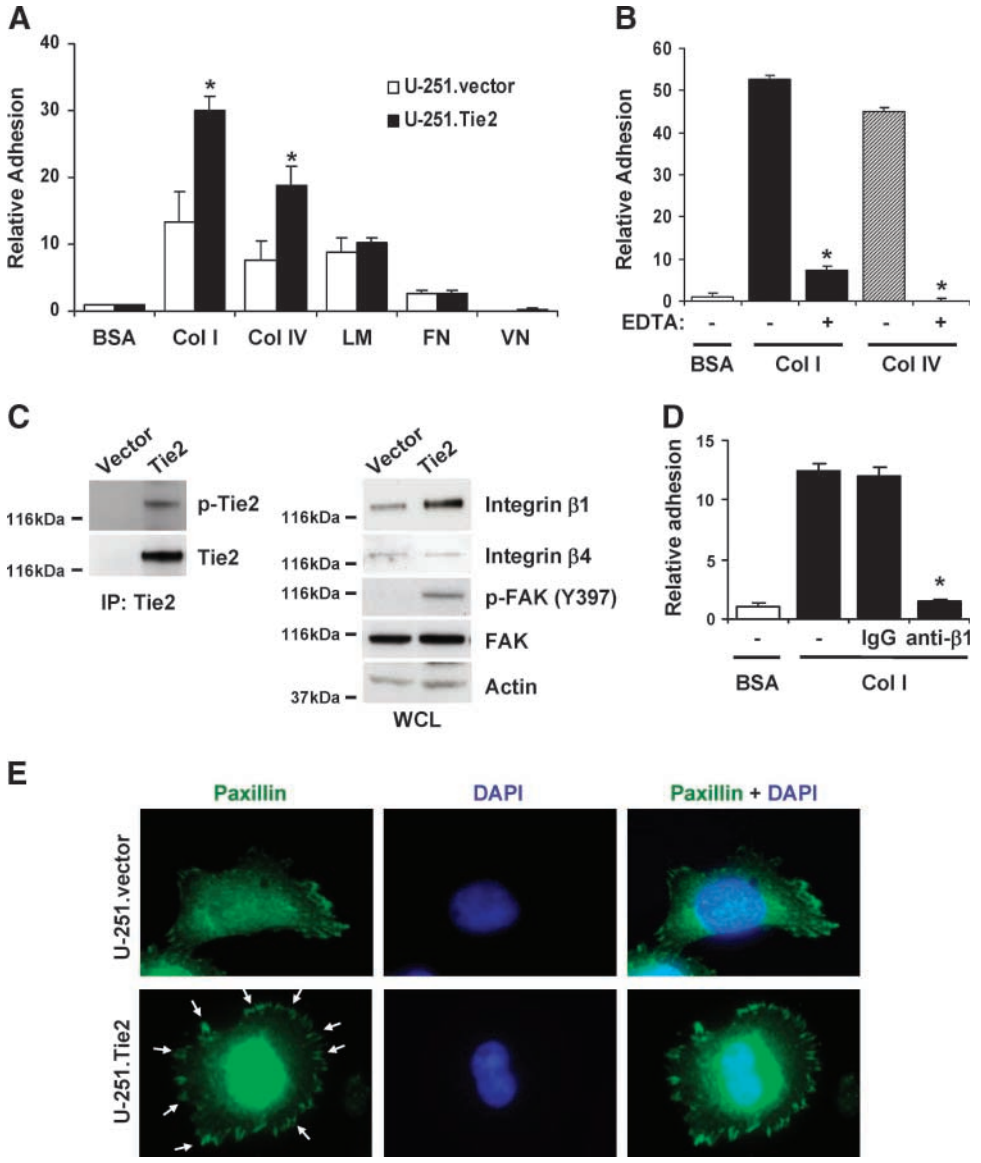

FIGURE 6. The presence of Tie2 is associated with the expression of integrin β1 and adhesion of glioma cells to ECM. **A.** Adhesion of U251.Tie2 cells (3×10^4 cells) to collagen I (*Col I*), collagen IV (*Col IV*), laminin (*LM*), fibronectin (*FN*), and vitronectin (*VN*; 10 nmol/L) was measured and compared with the adhesion of U251.vector cells. Results are expressed as the fold increase in the adhesion to every ECM component compared with BSA, equal to 1. *, $P < 0.005$ compared with U251.vector cells. **B.** Adhesion of U251.Tie2 cells (1×10^5 cells) to coatings of collagen I and IV (*Col I, Col IV*; 10 nmol/L) in the presence of EDTA (10 mmol/L) was measured. Results are represented as the fold increase in the adhesion of cells to ECM component-coated wells compared with BSA, equal to 1. *, $P < 0.001$ compared with adhesion of cells to ECM-coated wells in the absence of EDTA. **C.** Molecular modifications related to Tie2 expression. Expression pattern of total and phospho-Tie2, integrins β1 and β4, and total and phospho-FAK in U251.vector and U251.Tie2 cells. Whole cell lysates (*WCL*) or lysates obtained after Tie2 immunoprecipitation (*IP*) were assessed by Western blotting. Actin expression is shown as a loading control. **D.** Adhesion of glioma cells to collagen I (*Col I*) depended on integrin β1. U-251.Tie2 cells (3×10^4) were preincubated with anti-integrin β1 (anti-β1) or isotype (IgG) antibodies (10 μg/mL) for 10 min and plated onto wells coated with BSA or collagen I (10 nmol/L). Results are expressed as the fold increase in cell adhesion to collagen compared with BSA, equal to 1. *, $P < 0.001$ compared with isotype antibody-treated U-251.Tie2 cells. **E.** U-251.Tie2 or U-251.vector cells were allowed to attach to chamber slides coated with 50 μg/mL of collagen I for 3 h at 37°C. Cells were fixed, permeabilized, and incubated with anti-paxillin antibody followed by FITC-conjugated anti-mouse antibody. Arrows, focal adhesion contacts.

Establishment of U-251 MG Cells Expressing Tie2

U-251 MG cells were plated onto 12-well plates (5×10^4 cells/well) and 18 h later, transfected with 1 μg/well of empty pcDNA3 vector (Invitrogen, Carlsbad, CA) or pcDNA3-Tie2 plasmid (ref. 37; a generous gift from Dr. Bussolino, University of Torino, Italy) containing full-length human Tie2 cDNA using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Stably transfected clones were selected in growth medium containing 500 μg/mL of G418 (Sigma-Aldrich). After selection, U251.Tie2 clones were maintained in growth medium containing 300 μg/mL of G418.

Transfection with Tie2 siRNA Oligonucleotides and Tie2 shRNA Vector

We purchased Tie2 siRNA and a scrambled siRNA (negative control) from Santa Cruz Biotechnology. U-87 MG cells were

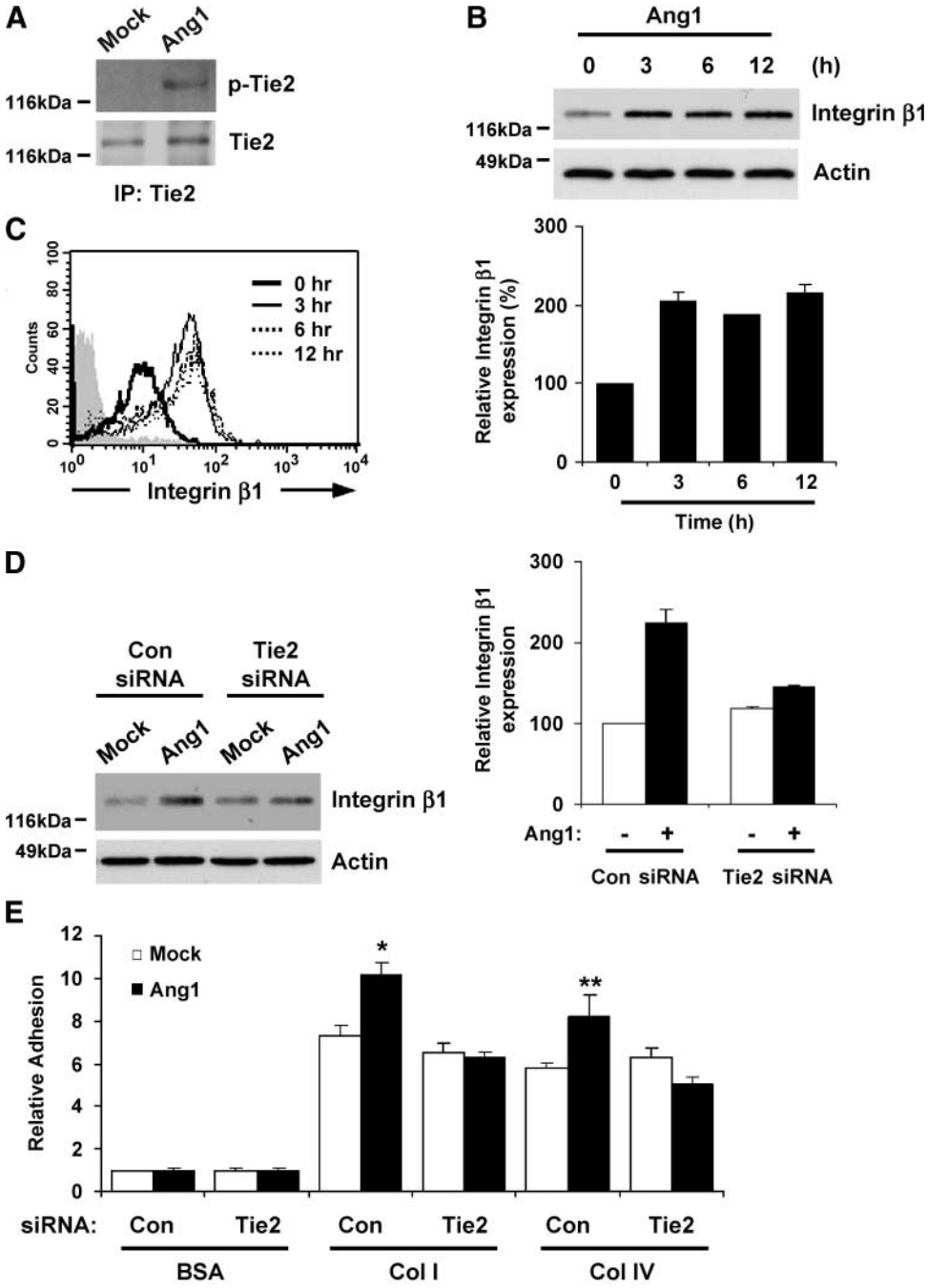

FIGURE 7. Ang1 signals through Tie2 to regulate integrin β1 and adhesion to ECM. **A.** Ang1 treatment resulted in Tie2 phosphorylation in U-87 MG cells. Tie2-immunoprecipitated lysates (*IP*) from serum-starved U-87 MG cells untreated or treated with Ang1 (500 ng/mL) for 10 min were analyzed by immunoblotting with anti-Tie2 and phosphotyrosine antibodies. **B** and **C.** Ang1 treatment resulted in up-regulation of integrin β1 in U-87 MG cells. **B.** Lysates from serum-starved U-87 MG cells untreated or treated with Ang1 (500 ng/mL) for the indicated times were analyzed by immunoblotting with anti-integrin β1 antibody. Representative experiment is shown. Actin expression was used as loading control (*top*). Relative integrin β1 level was quantified from three independent experiments as described in Materials and Methods. Signal intensity for integrin β1 was normalized to that of actin levels of expression for every sample, and graphed as relative to untreated sample (0 h; equal to 100%; *bottom*). **C.** Expression levels of integrin β1 in the cell surface were determined by flow cytometric analysis. Isotype IgG was used as negative control (*gray filled*). **D.** Knockdown of Tie2 expression inhibited Ang1-induced expression of integrin β1. U-87 MG cells were transfected with Tie2 siRNA or control siRNA (40 nmol/L). Serum-starved U-87 MG cells were stimulated with Ang1 (500 ng/mL) for 3 h. Left, cell lysates were analyzed by immunoblotting with anti-integrin β1 antibody. Representative experiment is shown. Actin expression is shown as the loading control. Right, relative protein levels were quantified as described in Materials and Methods. Signal intensity for integrin β1 was normalized to that of actin levels of expression for every sample, and graphed as relative to mock-treated samples (equal to 100%). **E.** Knockdown of Tie2 expression inhibited Ang1-mediated adhesion to collagen I (*Col I*) and collagen IV (*Col IV*). U-87 MG cells were transfected with Tie2 siRNA or control siRNA (40 nmol/L). Serum-starved U-87 MG cells were stimulated with Ang1 (500 ng/mL) for 3 h, and then 3×10^5 cells were plated on collagen I- or collagen IV-coated wells (10 nmol/L). Results are expressed as the fold increase in cell adhesion to collagen compared with BSA, equal to 1. *, $P < 0.005$; **, $P < 0.05$ compared with control siRNA-treated cells.

seeded at a density of 5×10^5 in 60 mm dishes 18 h before transfection. We did siRNA transfection (40 nmol/L) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Tie2 mRNA levels were analyzed by RT-PCR 48 to 72 h after siRNA transfection. We purchased pSHAG-MAGIC2 containing shRNA targeting Tie2 (clone V2MM_62361) and control vector pSHAG-MAGIC2 from Open Biosystems (Huntsville, AL). U-87 MG cells were transfected with control vector or Tie2 shRNA vector using Arrest-in transfection reagent (Open Biosystems) and stable cell line expression Tie2 shRNA was selected following the manufacturer's instructions.

Flow Cytometric Analysis

For the flow cytometric analysis, tumor-derived cell suspensions ($2\text{-}5 \times 10^5$ cells) were first treated with FcR blocking reagent (Miltenyi Biotec, Auburn, CA) to block unwanted binding of antibodies to the cells and then incubated with PE-conjugated mouse anti-human Tie2 (83715, R&D Systems), FITC-conjugated mouse anti-human CD31 (WM59), and APC-conjugated mouse anti-human CD34 (BD Biosciences, San Diego, CA) antibodies for 30 min at 4°C. To determine Tie2 expression in isogenic U-251 MG cells, Fc-blocked cells (5×10^5 cells) were stained with PE-conjugated mouse anti-human Tie2 antibody for 30 min at 4°C. To measure expression on the cell surface of β1, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ integrins, cells were incubated with mouse anti-human integrin β1, anti-human integrin $\alpha_v\beta_3$, or anti-human integrin $\alpha_v\beta_5$ antibodies (6S6, LM609, and P1F6, respectively, from Chemicon International, Temecula, CA) at 4°C for 1 h, and then cells were incubated with FITC-conjugated anti-mouse antibody (Santa Cruz Biotechnology) for 15 min at room temperature. We used PE- or FITC-conjugated mouse IgG_1 (BD Biosciences) as negative controls. Samples were analyzed using FACSCalibur (Becton Dickinson, San Jose, CA).

RT-PCR

Total cellular RNA (500 ng) was subjected to reverse transcription using a TITANIUM One-Step RT-PCR kit (Clontech, Mountain View, CA). After synthesis of cDNA at 50°C for 1 h, The PCR was done for 35 cycles consisting of the following steps: denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension at 68°C for 1 min. The sequences of primers for human Tie2 are published elsewhere (38). The sequences of the sense and antisense primers for human glyceraldehyde-3-phosphate dehydrogenase and Ang1 were as follows: glyceraldehyde-3-phosphate dehydrogenase (5′-ATGGGGAAGGTGAAGGTCGG-3′ and 5′-GACGGTGCCATGGAATTTGC-3′) and Ang1 (5′-CCACCTACAAGCTAGAGAAGCAAC-3′ and 5′-GACAAGGTTGTGGACTGTGTCC-3′).

Preparation of Membrane Fraction and Whole Cell Lysate

The total cellular membrane fraction was obtained from subconfluent cultures (150 mm dishes) as previously described (39). Cells were harvested and incubated for 10 min in homogenizing buffer [20 mmol/L Tris/HCl (pH 7.5), 5 mmol/L NaCl, 1 mmol/L EDTA, 5 mmol/L $MgCl_2$, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cells were sonicated at 10 W and 23 kHz for 20 sec (Sonic Dismembrator, Fisher Scientific, Houston, TX), and the suspension was centrifuged at 32,000 rpm for 1 h. Pellets were resuspended in 1 mL of homogenizing buffer and homogenized with a Dounce homogenizer (Kontes, Vineland, NJ), mixed with 0.1 mL of 10% Triton X-100 and 10 μL of 10% SDS, and then incubated for 30 min. Insoluble material was removed by centrifugation at 30,000 rpm for 1 h, and the supernatant was collected as the membrane fraction. Whole cell lysates were obtained by incubating cells in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 50 mmol/L NaF, 50 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, protease inhibitor mixture] for 30 min on ice.

Immunoprecipitation

To determine the phosphorylation of Tie2, cellular membrane proteins were precleared with protein A-agarose (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4°C. The supernatant was incubated with anti-Tie2 antibody (C-20; Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with protein A-agarose for 1 h at 4°C. Immunoprecipitates were recovered by centrifugation at 4,000 rpm for 3 min, washed, and solubilized in Laemmli sample buffer for SDS-PAGE and Western blot analysis.

Western Blot Analysis

Western blot analysis was conducted as described previously (40). Briefly, immunoprecipitates or whole cell lysates (15-40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-Tie2 (C-20; Santa Cruz Biotechnology), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti–epidermal growth factor receptor (1005; Santa Cruz Biotechnology), anti-integrin β1 (AB1952; Chemicon International), anti-integrin β4 (N-20; Santa Cruz Biotechnology), anti-phospho-FAK (Y397; Zymed Laboratories, South San Francisco, CA), anti-FAK (H-1; Santa Cruz Biotechnology), and anti-actin (C-11; Santa Cruz Biotechnology) antibodies. We visualized the membranes by electrochemiluminescence (Amersham Bioscience, Piscataway, NJ). Protein expressions were quantified by using NIH Image program (developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Immunofluorescence Studies

To examine Tie2 expression in neoplastic astrocytic cells, paraffin-embedded sections were incubated with anti-Tie2 (C-20, 1:100; Santa Cruz Biotechnology) and anti-GFAP (Z0334, 1:500; DakoCytomation, Carpinteria, CA) antibodies overnight at 4°C, and then with Texas red- or FITC-conjugated antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. To determine focal adhesion contacts, chamber slides (Nalge Nunc International, Naperville, IL) were coated with collagen type I (50 μg/mL) for 2 h at room temperature, blocked with 0.5% heat-inactivated bovine serum albumin (BSA) for 30 min, and washed twice with PBS before cells were added. Cells were then seeded onto the collagen-coated slides at

a density of 1×10^4. After incubation for 3 h at 37°C, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 5 min at 4°C, and blocked with PBS containing 10% goat serum and 1% BSA for 1 h at room temperature. Cells were then stained with mouse anti-paxillin antibody (5H11; Biosource, Camarillo, CA) for 1 h at room temperature. After cells were washed twice, they were further stained with FITC-conjugated anti-mouse antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Negative controls consisted of sections stained with the corresponding secondary antibodies without previous incubation with primary antibodies. Slides were mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and visualized with a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY). Images were captured using AxioVision Rel. 4.4 image software (Carl Zeiss).

Cell Adhesion Assay

For the cell adhesion assay, wells of a 96-well tissue culture plate were coated by exposure to the indicated concentrations of Ang1 (R&D Systems) and type I or IV collagen (BD Biosciences) for 2 h at room temperature and blocked with 0.5% BSA (heat-inactivated at 80°C for 10 min) in PBS for 30 min at room temperature. Cells were detached with trypsin/EDTA and rinsed in serum-free medium. The indicated number of cells were plated onto the wells, and the plate was incubated at 37°C for 1 h. Wells were gently washed with PBS, and attached cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich) for 10 min. Cells were solubilized in DMSO, and the absorbance of each well was read at 570 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). For the blocking experiments with EDTA or anti-integrin β1 antibody, cells were incubated with EDTA (10 mmol/L) or anti-integrin β1 antibody (10 μg/mL, 6S6; Chemicon International) for 10 min at 37°C, and cells were plated onto a 96-well plate in the presence or absence of EDTA or anti-integrin β1 antibody.

Statistical Analyses

In vitro data were analyzed using a two-tailed Student's *t* test. Data were expressed as the mean ± SD. Results from the human tissue array were evaluated using an exact Cochran-Armitage trend test.

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Molecular Cancer Research

Expression of the Receptor Tyrosine Kinase Tie2 in Neoplastic Glial Cells Is Associated with Integrin β 1-Dependent Adhesion to the Extracellular Matrix

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