The Small $\alpha_5\beta_1$ Integrin Antagonist, SJ749, Reduces Proliferation and Clonogenicity of Human Astrocytoma Cells

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Abstract

The potential role of $\alpha_5\beta_1$ integrins in cancer has recently attracted much interest. However, few $\alpha_5\beta_1$-selective antagonists have been developed compared with other integrins. The most specific nonpeptidic $\alpha_5\beta_1$ antagonist described thus far, SJ749, inhibits angiogenesis by affecting adhesion and migration of endothelial cells. We investigated the effects of SJ749 in two human astrocytoma cell lines, A172 and U87, which express different levels of $\alpha_5\beta_1$. SJ749 dose-dependently inhibited adhesion of both cell types on fibronectin. Application of SJ749 to spread cells led to formation of nonadherent spheroids for A172 cells but had no effect on U87 cell morphology. SJ749 also reduced proliferation of A172 cells due to a long lasting G_0-G_1 arrest, whereas U87 cells were only slightly affected. However, under nonadherent culture conditions (soft agar), SJ749 significantly reduced the number of colonies formed only by U87 cells. As U87 cells express more $\alpha_5\beta_1$ than A172 cells, we specifically examined the effect of SJ749 on A172 cells overexpressing α_5. Treatment of α_5-A172 cells with SJ749 decreased colony formation similarly to that observed in U87 cells. Therefore, in nonadherent conditions, the effect of SJ749 on tumor cell growth characteristics depends on the level of $\alpha_5\beta_1$ expression. Our study highlights the importance of $\alpha_5\beta_1$ as an anticancer target and shows for the first time that a small nonpeptidic $\alpha_5\beta_1$-specific antagonist affects proliferation of tumor cells. (Cancer Res 2006; 66(12): 6002-7)

Introduction

Gliomas are the most common deadly brain tumors, nowadays treated using conventional nontargeted therapies (surgery combined with radiotherapy followed by chemotherapy). Toxicity, poor blood brain barrier diffusion, and drug resistance are some of the reasons explaining the inefficiencies of current brain tumors treatment. New therapeutic strategies targeting multiple cell signaling pathways, such as inhibition of proliferation and migration or simultaneous inhibition of invasion and angiogenesis, need to be evaluated. Because integrins, $\alpha\beta$ heterodimeric transmembrane proteins, are involved in several of these processes, they could represent potential antitumoral targets. Thus, over the past decade, anticancer drug development focused on $\alpha_v\beta_3/\beta_5$ integrins resulted in several selective antagonists (1).

Studies on mice lacking α_v integrins reported extensive vasculogenesis, angiogenesis, and cerebral hemorrhage, suggesting antiangiogenic properties of $\alpha_v\beta_3/\beta_5$ (2, 3). However, among the different integrins, the fibronectin receptor $\alpha_5\beta_1$ now seems as the unique and unambiguously proangiogenic integrin (4). It is overexpressed in angiogenic endothelial cells but also in astrocytomas and glioblastomas (5) and may represent a new anticancer target. To date, few molecules acting as $\alpha_5\beta_1$ antagonists have been developed, with SJ749 being the most selective nonpeptidic $\alpha_5\beta_1$ antagonist described thus far (6–9). SJ749 was designed as a fibronectin adhesion antagonist that specifically inhibits $\alpha_5\beta_1$-mediated cell adhesion to fibronectin. However, the characterization of integrins as activators of specific intracellular signals opens now new ways to consider adhesion antagonists as potential modulators of cell signaling (10). Indeed, adhesion antagonists behave as competitive inhibitors but also as ligand mimetics (11) and may convey similar stimuli to the cell as for endogenous integrin ligands. This is of importance as the resistance of tumor cells to anoikis allows them, under nonadherent conditions, to invade surrounding tissues and vessels during metastasis.

SJ749 inhibits endothelial cell adhesion to fibronectin and *in vivo* angiogenesis (6), but its ability to affect directly tumor cells is currently unknown. Therefore, we evaluated the effects of SJ749 on cell adhesion and proliferation under adherent and nonadherent conditions in two human astrocytoma cell lines, A172 and U87, respectively, described as grade 4 and 3. A172 cells express less $\alpha_5\beta_1$ than U87 cells, with A172 expressing mostly $\alpha_5\beta_1$. We found that SJ749 behaves as an efficient fibronectin adhesion antagonist in both cell lines, but differential effects were observed in terms of proliferation and cell cycle on adherent cells and clonogenicity of nonadherent cells. Our study shows that the level of $\alpha_5\beta_1$ expression in nonadherent tumor cells plays a critical role in the antagonistic effects of SJ749. It also confirms the importance of $\alpha_5\beta_1$ as an anticancer target and shows that a small nonpeptidic $\alpha_5\beta_1$-specific antagonist can effectively affect proliferation and clonogenicity of tumor cells.

Materials and Methods

Material. SJ749 ((S)-2-[(2,4,6-trimethylphenyl) sulfonyl] amino-3-[7-benzyloxycarbonyl-8-(2-pyridinylaminomethyl)-1-oxa-2,7-diazaspiro-(4,4)-non-2-en-3-yl] carbonylamino] propionic acid) was synthesized as described in the patent WO 97/33887. Rabbit polyclonal anti-α_5 (AB1928) and anti-β_1 (AB1952) integrin antibodies were from Chemicon (Euromedex, Souffelweyersheim, France); propidium iodide, fibronectin, and collagen were from Sigma (L'Isle d'Abeau Chesnes, France); vascular cell adhesion molecule 1 (VCAM1) was from R&D Systems (Lille, France); and CellTiter96 Aqueous One Solution Cell proliferation assay was from Promega (Charbonnières les Bains, France).

RNA extraction and reverse transcription-PCR. Total cellular RNA was extracted following the manufacturer's instructions using the

Note: In memory of Dr. P. Klotz, deceased June 2005.

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TriReagent (Euromedex). Reverse transcription-PCR (RT-PCR) was done as described previously (12). The following sets of primers (Invitrogen, Cergy-Pontoise, France) were used for PCR: β-actin (428 bp) forward (5′-GGTCAGAAGGATTCCTATGT-3′) and reverse (5′-ATGAGGTAGTCAGTCAGGTC-3′), α_5 (584 bp) forward (5′-AGCCTGTGGAGTACAAGTCC-3′) and reverse (5′-AAGTAGGAGGCCATCTGTTC-3′), and β_1 (756 bp) forward (5′-AGCAGGGCCA AATTGTGGGT-3′) and reverse (5′-CCACCAAGTTTCC-CATCTCC-3′). The total number of cycles was 30 for β-actin and 35 for α_5 or β_1 (denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds). The amplification products were visualized on a 1.5% ethidium bromide–stained agarose gel.

Cell culture and transfection. U87 and A172 cell lines were from American Type Culture Collection, Manassas, VA. Cells were cultured in Eagle's MEM supplemented with 10% heat-inactivated FCS, 0.6 mg/mL glutamine, 200 IU/mL penicillin, 200 IU/mL streptomycin, and 0.1 mg/mL gentamicin. Human α_5 cDNA of the pECE-α_5 integrin plasmid (provided by Dr. E. Ruoslahti, The Burnham Institute, La Jolla, CA) was subcloned in the *Xba*I/*Not*I sites of the expression vector pcDNA3.1 (Invitrogen). α_5-pcDNA3.1 or mock pcDNA3.1 (1 μg) was transfected using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. Transfected cells were selected using G418 (1 mg/mL) and screened for α_5 expression by immunoblotting.

Western blot. After collection, cells were lysed in buffer A (1% Triton X-100, 100 mmol/L NaF, 10 mmol/L NaPPi, 1 mmol/L Na_3VO_4 in PBS supplemented with complete anti-protease cocktail; Roche, Meylan, France) 1 hour at 4°C. Protein (20 μg) was electrophoresed by SDS-PAGE (8% polyacrylamide), and separated proteins were transferred on polyvinylidene difluoride (PVDF) membranes (Amersham, Orsay, France). Blots were probed with anti-α_5 and anti-β_1 antibodies (0.5-1 μg/mL) followed by horseradish peroxidase–conjugated anti-rabbit antibody (1/100,0000). The enhanced chemiluminescence system (Amersham) followed by exposure to CL-Xposure films (Kodak, Rochester, NY) was used to visualize proteins.

Adhesion assays. Cells were plated in the presence of solvent (50% methanol/50% H_2O) or SJ749 (10^{-9} to 10^{-5} mol/L) on fibronectin (5 μg/mL), collagen (10 μg/mL), or VCAM1-coated (10 μg/mL) 96-well plates and incubated for 30 minutes. Adhesion was measured after coloration with crystal violet by absorbance recording at 595 nm (13). IC_{50}s were calculated using GraphPad Prism software.

Proliferation assays. Cells were plated in 96-well plates in 2% serum-containing medium and left overnight until adhesion. They were treated 0, 24, 48, and 72 hours with solvent (50% methanol/50% H_2O) or SJ749 (10 μmol/L). Alternatively, cells were plated on poly-HEMA-coated 96-well plates (nonadherent conditions) in 2% serum-containing medium for 72 hours. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (Promega, France) according to the manufacturer's instructions.

Soft agar assays. Cells were seeded into soft agar as described previously (14) and treated for 14 days with solvent (50% methanol/50% H_2O) or SJ749 (10 μmol/L). Colonies formed were stained with 0.005% crystal violet and counted. Results are expressed as percentage colonies formed in presence of SJ749 versus solvent.

Cell cycle analysis. Cells were plated in 24-well plates (10,000 per well) and treated for 72 hours with solvent (50% methanol/50% H_2O) or SJ749 (10 μmol/L). After collection, cells were resuspended in 300 μL hypotonic fluorochrome solution (5 μg propidium iodide, 3.4 mmol/L sodium citrate, and 0.1% Triton X-100 in PBS). DNA content was analyzed by flow cytometry (Becton Dickinson FACScan, San Diego, CA). Ten thousand events per sample were acquired, and cell cycle repartition was determined using ModFit software.

Statistical analysis. Data are represented as mean ± SE. In all cases, *n* refers to the number of independent experiments. Statistical analyses were done by the Student's *t* test. $P < 0.05$ was considered significant.

Results and Discussion

Characterization of integrin subtypes in astrocytoma cell lines. As substrate specificity is determined by integrins, A172 and U87 cells were plated on fibronectin, collagen, and VCAM1 to identify the nature of expressed integrins. A172 cells only adhered on fibronectin in contrast to U87 cells, which attached equally to all substrates (Fig. 1*A*). The incapacity of A172 to attach on collagen and VCAM1 excludes the presence of their respective receptors ($\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_{IIb}\beta_3$ for collagen and $\alpha_4\beta_1$ or $\alpha_4\beta_7$ for VCAM1) and suggests that A172 cells predominantly express the fibronectin receptor $\alpha_5\beta_1$. The ability of U87 cells to adhere to various substrates indicates that they express a larger panel of integrins. A172 adhesion to fibronectin was sensitive to the GRGDS peptide and insensitive to the scramble control peptide SDGRG, confirming also the involvement of RGD-sensitive integrins (data not shown). In any case, the fibronectin receptor appeared present in both cell lines, and we therefore determined the level of expression of $\alpha_5\beta_1$.

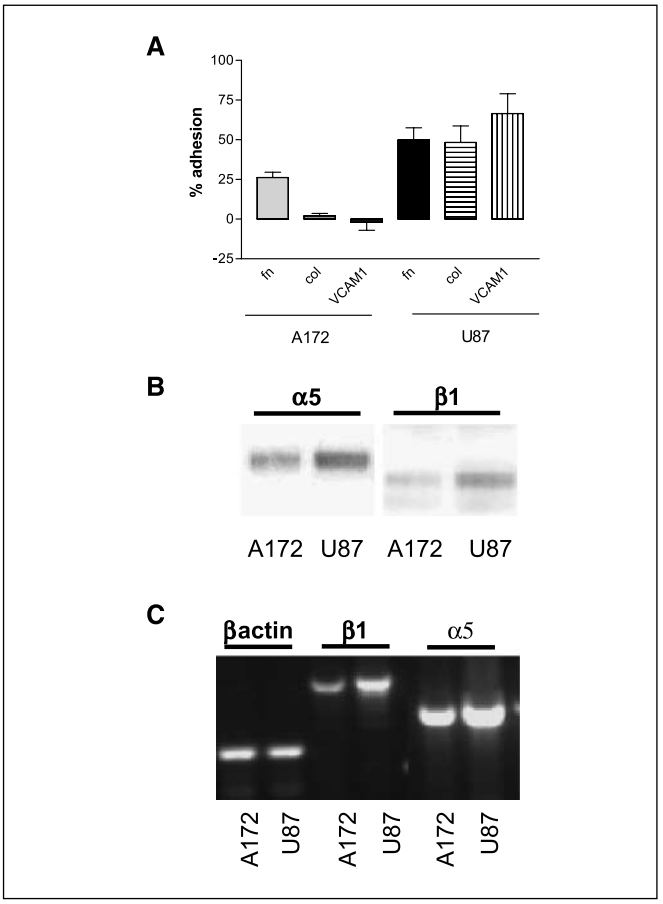

Figure 1. Characterization of $\alpha_5\beta_1$ integrin in A172 and U87 cells. *A,* adhesion assays. Cells were plated in 96-well plates coated with fibronectin (*fn*; 5 μg/mL), collagen (*col*; 10 μg/mL), or VCAM1 (10 μg/mL). After 30 minutes of incubation followed by three washes, adherent cells were measured by coloration with crystal violet and absorbance recording at 595 nm. *Columns,* mean of three experiments in sextuplicates; *bars,* SE. *B,* Western blot analysis. Total protein extracts of A172 and U87 cells were resolved by SDS-PAGE, and PVDF membranes were probed with anti-α_5 or anti-β_1 integrin antibodies. *C,* RT-PCR analysis. β-Actin (428 bp), α_5 integrin (584 bp), and β_1 integrin (756 bp) transcripts in A172 and U87.

The presence of α_5 and β_1 subunits was checked at the protein and RNA level. As expected, α_5 and β_1 subunits were present in A172 and U87 cells as shown by Western blots (Fig. 1*B*). α_5 and β_1 were about twice more abundant in U87 than in A172 cells (1.7 ± 0.3 and 1.5 ± 0.2 times more for β_1 and α_5 integrin, respectively, in U87 compared with A172 cells; $n = 6$). These results were confirmed

by RT-PCR (Fig. 1*C*). Although $\alpha_5\beta_1$ integrins are crucial in tumoral angiogenesis and SJ749, an $\alpha_5\beta_1$ antagonist, reduces adhesion and migration of endothelial cells (6), this drug has not yet been tested on tumor cells. We therefore determined its effects on astrocytoma cells, which express different levels of $\alpha_5\beta_1$.

SJ749 inhibits adhesion of A172 and U87 cells to fibronectin and induces the appearance of A172-floating spheroids. SJ749 dose dependently inhibited adhesion of A172 (IC_{50}, 84 $\pm$ 2 nmol/L; n = 6) and U87 (IC_{50}, 630 $\pm$ 2 nmol/L; n = 5) cells on fibronectin (Fig. 2*A, left*). In contrast, SJ749 was inefficient in blocking U87 adhesion on collagen (Fig. 2*A*, *left*), confirming the specificity of SJ749 for $\alpha_5\beta_1$. Compared with previously reported IC_{50}s of 300 to 800 nmol/L (6), SJ749 was more potent on A172 cells. When added to adherent cells cultured as monolayers, SJ749 induced detachment, which was accompanied by striking morphologic changes of A172 cells. After 24 hours of exposure to the drug, A172 cells rounded up and detached to form clusters termed spheroids (Fig. 2*A, right*). Similar effects have been described for colon carcinoma cells treated with a small $\alpha_v\beta_3$ antagonist (15). In contrast, fewer U87 cells detached and did not form spheroids (Fig. 2*A, right*).

SJ749 reduces the proliferation of adherent A172 and U87. When added on cell monolayers, SJ749 significantly decreased proliferation of both cell lines. In contrast with U87 cells, a time dependence was observed in A172 cells (20%, 35%, and 40% inhibition after 24, 48, and 72 hours, respectively, for A172 cells versus 20% inhibition at any time tested for U87 cells; Fig. 2*B, left*). Analysis of the cell cycle progression revealed an accumulation of A172 cells in G_0-G_1 phase (Fig. 2*B, right*) visible after 24 hours of treatment with SJ749 and maintained up to 72 hours (Fig. 2*B, right*). Concomitantly, a decrease in S and G_2-M phases is observed compared with solvent-treated cells (data not shown). Although SJ749 induced an accumulation of U87 cells in G_0-G_1 phase visible after 12 hours of treatment, it seems inefficient to block U87 cell cycle progression for more than 24 hours (Fig. 2*B, right*). No significant apoptosis was detected in A172 cells before 72 hours of treatment (2.5 $\pm$ 0.9% versus 14.5 $\pm$ 6.8% for solvent- and SJ749-treated cells, respectively), and no apoptosis was observed in U87 cells at any time tested.

To determine if the level of $\alpha_5\beta_1$ influences cell response to the drug, A172 cells were transfected with the α_5 integrin subunit so that the level expressed equals that of U87 cells (data not shown). Overexpression of α_5 in A172 cells led to 25% more adhesion to fibronectin compared with nontransfected cells (mock-A172), thus reaching the level observed in U87 cells (Fig. 3*A*). No adhesion to collagen was found, suggesting that α_5 overexpression did not modulate other substrate receptors (Fig. 3*A*). Although SJ749 dose dependently reduced adhesion of α_5-A172 to fibronectin (IC_{50}, 3 μmol/L), it was less potent than on A172 cells (Fig. 3*B, left*). As reported previously for A172 cells (Fig. 2*A*), SJ749 induced the detachment of α_5-A172 cells forming floating spheroids (Fig. 3*B, right*). Although mock and α_5-A172 proliferation was reduced in a similar manner by SJ749 until 48 hours, their proliferation rate changed after that time point with mock-A172 proliferating slower than α_5-A172 (Fig. 3*C, left*). Accordingly, mock-A172 progression in the cell cycle was reduced as reported previously for A172 (Fig. 2*B*). SJ749 induced an accumulation in G_0-G_1 phase visible after 24 hours and maintained up to 72 hours (Fig. 3*C, left*). In contrast, α_5-A172 cell cycle progression was only reduced after 24 hours of treatment with SJ749 (Fig. 3*C, right*). Altogether, data suggest that in adherent conditions, α_5-A172 cells behave similarly to U87 in response to SJ749 in terms of sensitivity, proliferation, and cell cycle progression, although SJ749 still induced detachment of α_5-A172.

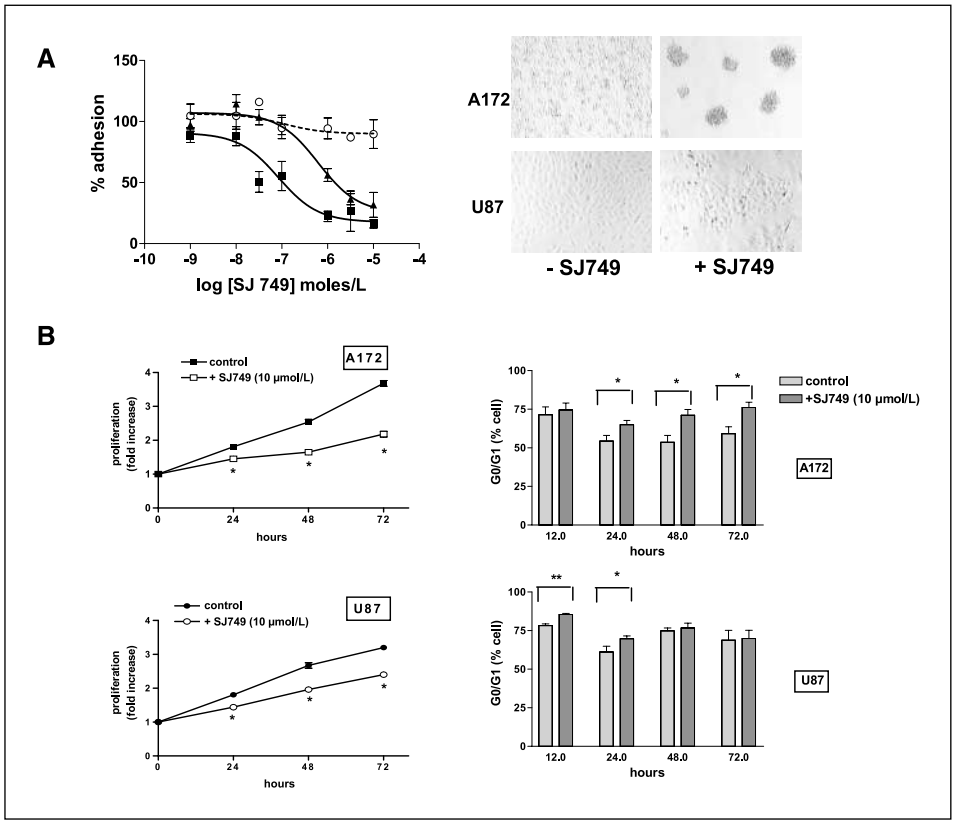

Figure 2. Effects of SJ749 on adhesion, cell morphology, proliferation, and cell cycle. *A, left,* effects of SJ749 on cell adhesion. Cells were plated in 96-well plates coated with fibronectin (5 μg/mL) or collagen (10 μg/mL) for 30 minutes in the presence or absence (100% adhesion) of increasing concentrations of SJ749. ■, A172 cells on fibronectin; ▲ and ○, U87 cells on fibronectin and collagen, respectively. *Points,* mean of two to five experiments in sextuplicates; *bars,* SE. *Right,* effects of SJ749 on cell morphology. Spread A172 and U87 cells were treated for 24 hours with solvent or SJ749 (10 μmol/L) in 2% serum medium before microscopic observation. *B, left,* proliferation assay. Fold increase in proliferation of A172 and U87 treated for 24, 48, and 72 hours with solvent or SJ749 (10 μmol/L) versus nontreated cells at day 0 (n = 3 in sextuplicates; *, $P < 0.05$). *Right,* cell cycle analysis. A172 and U87 monolayer cells were treated with solvent or SJ749 (10 μmol/L) for 12, 24, 48, and 72 hours before the analysis of the cell cycle by flow cytometry. Percentage of cells present in G_0-G_1 phase (n = 4-5 in duplicate; *, $P < 0.05$, **, $P < 0.01$).

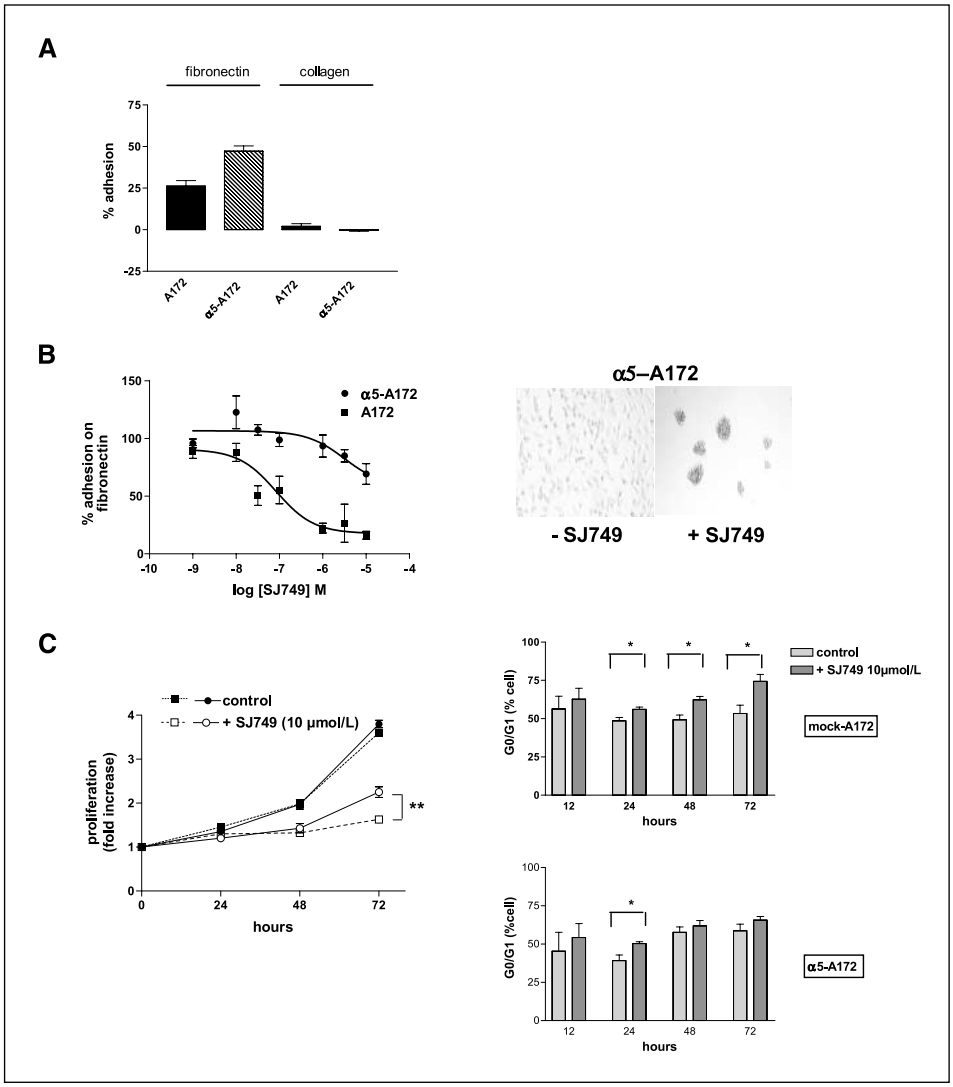

Figure 3. Effects of SJ749 on α_5-A172 adhesion, cell morphology, proliferation, and cell cycle. *A,* adhesion assays. Mock-A172 or α_5-A172 was plated on fibronectin- (5 μg/mL) or collagen-coated (10 μg/mL) wells, and adhesion was measured after 30 minutes (n = 2-5 in sextuplicates). *B, left,* effects of SJ749 on cell adhesion. Cells were plated in 96-well plates coated with fibronectin (5 μg/mL) for 30 minutes in the presence or absence (100% adhesion) of increasing concentrations of SJ749 [A172 (■) and α_5-A172 (●); n = 4 in sextuplicates]. *B, right,* effect of SJ749 of cell morphology. α_5-A172 was treated with solvent or SJ749 (10 μmol/L) for 24 hours before microscopic observation. *C, left,* proliferation assay. Fold increase in proliferation of mock-A172 (□ and ■) and α_5-A172 (○ and ●) treated for 24, 48, and 72 hours with solvent or SJ749 (10 μmol/L) versus nontreated cells at day 0 (n = 3 in sextuplicates; *, $P < 0.05$). *B, right,* cell cycle analysis. Mock-A172 and α_5-A172 monolayer cells were treated with solvent or SJ749 (10 μmol/L) for 12, 24, 48, and 72 hours before the analysis of the cell cycle by flow cytometry. Percentage of cells present in G_0-G_1 phase (n = 5 in duplicate; *, $P < 0.05$; **, $P < 0.01$).

Cell proliferation in nonadherent conditions. To determine if the transient inhibitory effect of SJ749 on U87 proliferation and cell cycle progression was linked to the inefficacy of the drug to detach these cells, proliferation of A172 and U87 was determined in nonadherent conditions in absence of SJ749. As already reported for tumor cells (16), both cell lines formed spheroids growing slower (A172) or not (U87) compared with adherent conditions (Fig. 4*A, left* versus Fig. 2*B, right*). Suspension induced significant apoptosis of U87 (Fig. 4*A, right*) without affecting A172 (data not shown).

In the absence of SJ749 but under nonadherent conditions, no modifications of the cell cycle progression were observed in A172 and mock-A172 cells when compared with adherent conditions (Fig. 4*B, left*). As no apoptosis and no modifications of the cell cycle were observed, it seems that the reduction in proliferation observed when A172 cells are in suspension might be the result of a slowed cell cycle progression. In contrast, U87 and α_5-A172 cells showed an increase of G_0-G_1 phase visible for U87 as early as 12 hours and maintained up to 72 hours and for α_5-A172 cells as early as 24 hours until 48 hours (Fig. 4B, *right*). In contrast to A172 and mock-A172, suspension alone triggered cell cycle blockage of U87 and α_5-A172 cells. It also revealed an increased sensitivity toward matrix attachment for cell cycling in these cells.

Altogether, data showed contrasting effects of SJ749 on the different cell lines. SJ749 not only inhibits adhesion but also exerts a proper inhibitory effect in the cell cycle progression of A172 and mock-A172 cells. U87 cells are less sensitive to SJ749 in terms of detachment but dependent on matrix attachment for cell cycling and survival. Therefore, the reduction in U87 proliferation observed in presence of SJ749 might be due to an early blocking of the cell cycle appearing after 12 hours of treatment until 24 hours. Even if able to detach α_5-A172 cells, SJ749 is unable to affect sustainably their cell cycle. These results indicate that (*a*) the effects of SJ749 on A172, mock-A172, and α_5-A172 adhesion and cell cycle progression are separate events, (*b*) the overexpression of α_5 subunit conferred some U87 characteristics to A172 cells, and (*c*) counter-regulatory cell cycle pathways may exist in α_5-A172 cells that mask the effects of SJ749 after detachment. Future work is needed to address this last hypothesis.

Effects of SJ749 on cell clonogenicity. Finally, to characterize the effects of SJ749 on tumorigenicity and invasiveness of astrocytoma cells, anchorage-independent growth in soft agar (14) in the presence of solvent or SJ749 was undertaken. In the presence of solvent, α_5-A172 cells formed significantly more colonies than A172 (data not shown), suggesting that increased α_5 levels led to a

more aggressive phenotype. This agrees with studies reporting an increase or decrease in cancer cell tumorigenicity and invasiveness after α_5 overexpression (5, 17–19). In presence of SJ749, the number of colonies formed by U87 and α_5-A172 was significantly reduced and remained unaffected for the others (Fig. 4*C*). Overall, SJ749 was unable to completely abrogate the increased number of colonies obtained for α_5-transfected cells (30% increase versus 10% decrease of colonies in the presence of SJ749), probably due to the heterogeneous population of transfected cells.

Therefore, high levels of α_5 renders cells more sensitive to SJ749 with the acquisition of U87 properties probably due to the increased sensitivity to matrix attachment for cell cycling. It also supports the crucial role of α_5 in the anticlonogenic effect of SJ749, confirmed by the fact that β_1-A172 cells are insensitive to SJ749 (Fig. 4*C*).

Conclusions

We found that SJ749 differently affects human astrocytoma cell lines depending on both state of adhesion and level of α_5 expression. The degree of SJ749 inhibition of adhesion and clonogenicity was clearly dissociable. The former effect seemed inversely correlated to the level of integrin expression, whereas the latter seemed dependent on a threshold level of integrin expression. This suggests that SJ749 binding to $\alpha_5\beta_1$, even when sufficient to compete with fibronectin for adhesion, requires higher integrin expression to inhibit cell growth in anchorage-independent culture. This may be related to the differences between inhibition of activated pathways (through inhibition of adhesion) and activation of inhibitory pathways (through nonligated integrin in nonadherent conditions). These effects of SJ749 on tumor cells contrast with those of another $\alpha_5\beta_1$ antagonist, the peptide Ac-PHSCN-NH2 (ATN161) that, without inhibiting cell adhesion, has anti-invasive, antiangiogenic, and antimetastatic activities. ATN161 mainly targets endothelial cells, as no cell cycle changes or apoptosis of tumor cells was reported (20–22).

We show for the first time that a small nonpeptidic $\alpha_5\beta_1$ antagonist directly affects cerebral tumor cells by modulating cell cycle progression and clonogenicity. Our results support that $\alpha_5\beta_1$ integrins are potential anticancer targets. Strategies for discovering

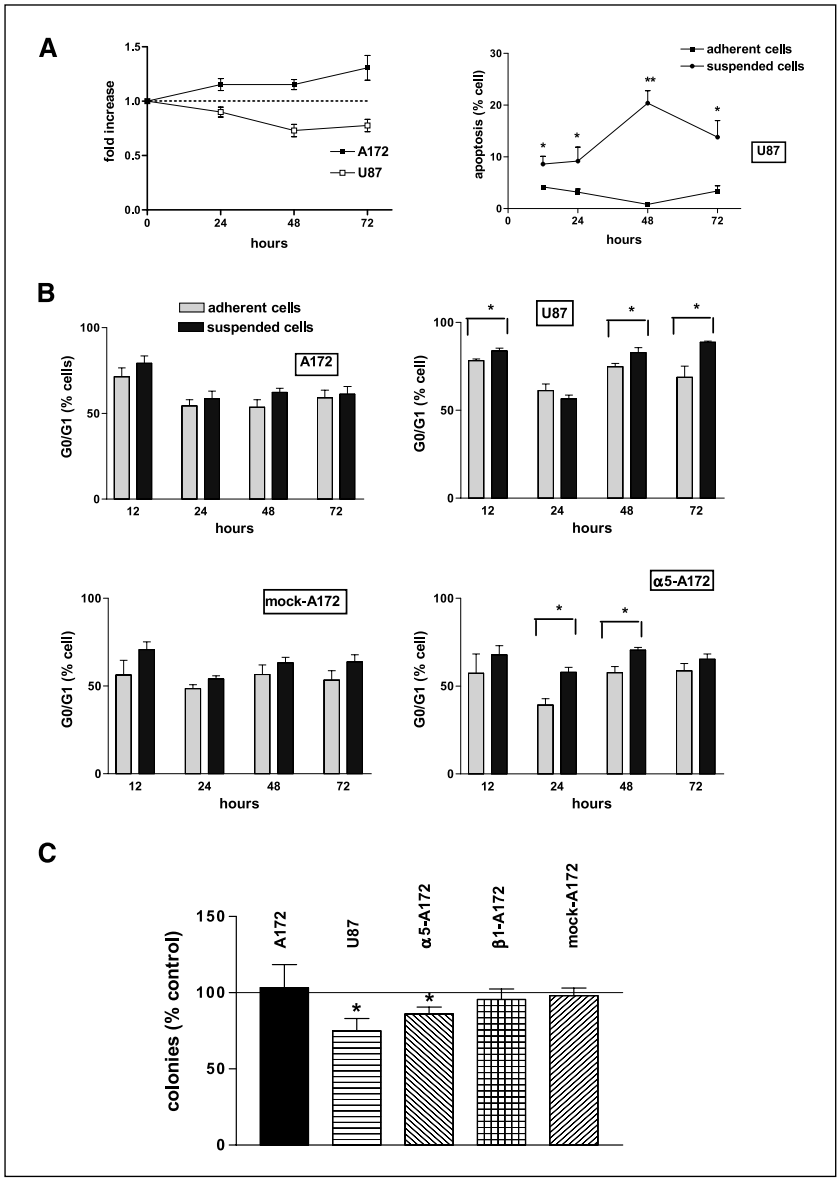

Figure 4. Proliferation under nonadherent cell culture conditions. *A, left,* proliferation assay. Time course analysis of proliferation of A172 and U87 cells on polyHEMA-coated plates from 0 to 72 hours (n = 3 in sextuplicates). Fold increase in proliferation of A172 and U87 grown for 24, 48, and 72 hours on polyHEMA versus cells at day 0. *A, right,* apoptosis of U87 cells on polyHEMA-coated plates. Percentage of apoptosis (sub-G_1 phase) of U87 cells grown under adherent and nonadherent conditions determined by flow cytometry (n = 4-5 in duplicate; *, $P < 0.05$; **, $P < 0.01$). *B,* cell cycle analysis. Cells were grown on polyHEMA for 12, 24, 48, and 72 hours before the analysis of the cell cycle by flow cytometry. Percentage of cells present in G_0-G_1 phase (n = 5 in duplicate; *, $P < 0.05$). *C,* colony formation in soft agar. A172, U87, α_5-A172, β_1-A172, and mock-A172 were grown in soft agar for 14 days in the presence of solvent or SJ749. Histograms show the number of colonies formed in the presence of drug as percentage of control in the presence of solvent (n = 3-12 in triplicate; *; $P < 0.05$, paired t test).

new integrin antagonists, which inhibit tumor cells, should be based on inhibition of not only cell adhesion but also integrin-dependent cell signaling. A therapeutic approach using such $\alpha_5\beta_1$ antagonists would then combine antiangiogenic and antitumoral effects.

Acknowledgments

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