Downregulation of integrin $\alpha_\nu\beta_3$ expression and integrin-mediated signaling in glioma cells by adenovirus-mediated transfer of antisense uPAR and sense p16 genes

Yoshiaki Adachi, Sajani S. Lakka, Nirmala Chandrasekar, Niranjan Yanamandra, Christopher S. Gondi, Sanjeeva Mohanam, Dzeng H. Dinh, William C. Olivero, Meena Gujrati, Takashi Tamiya, Takashi Ohmoto, Bharat Aggarwal and, Jasti S. Rao

Division of Cancer Biology, Departments of Biomedical Therapeutic Sciences (S.S.L., N.C., N.Y., C.S., G S.M., J.S.R), Neuropathology (M.G.), Neurosurgery (D.H.D., W.C.O., J.S.R) University of Illinois College of Medicine at Peoria, Peoria, IL.61656, Department of Bio-immunotherapy, (B.A) M. D. Anderson Cancer Center Houston TX 77030 and Department of Neurological Surgery (YA, T.T. and T.O), Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan.

Running title: $\alpha_\nu\beta_3$ expression and Integrin mediated signaling in gliomas.

Keywords: alphaVbeta3 integrin; urokinase type plasminogen activator receptor; p16; adenovirus; glioma

Correspondence should be addressed to J.S.Rao: Division of Cancer Biology, Departments of Biomedical and therapeutic Sciences and Neurosurgery, UIC, college of Medicine at Peoria, Peoria II.61656.email: jrao@uic.edu
Abstract

Interaction between the extracellular matrix and integrin receptors on cell surfaces leads not only to cell adhesion but also to intracellular signaling events that affect cell migration, proliferation, and survival. The vitronectin receptor $\alpha v\beta 3$ integrin is of key importance in glioma cell biology. The expression of urokinase-type plasminogen activator receptor (uPAR) was recently shown to co-regulate with the expression of $\alpha v\beta 3$ integrin; moreover, restoration of the p16 protein in glioma cells inhibits the $\alpha v\beta 3$ integrin-mediated spreading of those cells on vitronectin. Thus we hypothesized that adenovirus-mediated downregulation of uPAR and overexpression of p16 might downregulate the expression of $\alpha v\beta 3$ integrin and the integrin-mediated signaling in glioma cells, thereby defeating the malignant phenotype. In this study, we used replication-deficient adenovirus vectors that contain either a uPAR antisense expression cassette (Ad-uPAR) or wild-type p16 cDNA (Ad-p16) and a bicistronic adenovirus construct in which both the uPAR antisense and p16 sense expression cassettes (Ad-uPAR/p16) are inserted in the E1-deleted region of the vector. Infecting the malignant glioma cell line SNB19 with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 in the presence of vitronectin resulted in decreased $\alpha v\beta 3$ integrin expression and integrin-mediated biological effects, including adhesion, migration, proliferation, and survival. Our results support the therapeutic potential of simultaneously targeting uPAR and p16 in the treatment of gliomas.
Introduction

Malignant gliomas are the most common primary brain tumors in adults and children and are refractory to conventional forms of therapy (1). Because >90% of glioblastoma recurrences occur at the margin of the original tumor (2), the biochemical conditions unique to the malignant glioma margin are thought to confer a survival advantage to tumor cells. Gliomas have been shown to express vitronectin (VN), an extracellular matrix (ECM) protein, with the greatest amounts present at the tumor margin; in contrast, the normal adult cortex and white matter are devoid of VN (3).

Malignant glioma cells also express the two cognate receptors for VN, the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. The $\alpha_v\beta_3$ integrin heterodimer is particularly expressed by glioma cells at the advancing tumor margin (3). Integrins are cell-surface receptors that mediate the physical and functional interactions between a cell and its ECM. Although the classic role of integrins is to anchor cells to the ECM, integrins have many other functions in addition to adhesion. Interaction between the ECM and cell-surface integrins has been shown to lead to intracellular signaling events that affect cell migration, proliferation, and survival (4, 5). The $\alpha_v\beta_3$ integrin has been identified as being of key importance in various normal and malignant cell types (6,7) including glioma (8) and thus may be an anti-tumor therapeutic target. Indeed, Cheresh’s group has used the LM609 anti-$\alpha_v\beta_3$ heterodimer antibody to produce tumor regression in in vitro and in vivo models (7, 9, 10). More recently, peptido-mimetic inhibitor selective for the $\alpha_v\beta_3$ integrin heterodimer has also demonstrated anti-tumor effects in germ cell tumors (11,12) and in gliomas (13).
The urokinase-type plasminogen activator receptor (uPAR) is a single-chain, highly glycosylated protein with a molecular mass of 50,000–60,000 that is anchored on the cell membrane by a glycosylphosphatidylinositol moiety (14). Urokinase-type plasminogen activator (uPA) binds to uPAR and catalyzes the conversion of inactive plasminogen into plasmin, which then degrades a variety of ECM proteins and activates metalloproteinases and growth factors (15, 16). uPAR also has been shown to regulate integrin function (17), and the expression of uPAR mRNA is co-regulated with that of \( \alpha v \beta 3 \) mRNA (18). Our studies and others showed that uPAR levels were significantly increased during the progression of human gliomas (19, 20) and tumor formation and tumor growth was inhibited in antisense uPAR clones (21, 22).

In gliomas, the p16 tumor suppressor gene is frequently inactivated (23, 24). The 16,000-Da p16 protein acts as a cyclin-dependent kinase (cdk) inhibitor, inhibiting the binding of the cdk4 and cdk6 proteins to cyclin D1. Recent reports indicate that restoration of p16 protein inhibited \( \alpha v \beta 3 \) integrin-mediated cell spreading on VN (25).

On the basis of these reports, we hypothesized that downregulation of uPAR and overexpression of p16 through the use of adenovirus vectors might cause the downregulation of \( \alpha v \beta 3 \)-integrin expression in glioma cells. Moreover, if the \( \alpha v \beta 3 \)-mediated signaling between VN and the glioma cells were also down-regulated, this strategy could defeat the malignant phenotype of those cells.

We have already reported the generation of replication-deficient adenovirus vectors that contain a uPAR antisense expression cassette (Ad-uPAR) (26) or a p16 sense expression cassette (Ad-p16) (27). We have also generated a bicistronic adenovirus construct (Ad-uPAR/p16) in which the uPAR antisense and p16 sense expression
cassettes are inserted in the E1-deleted region of the vector. In this study, we cultured the SNB19 glioma cell line in the presence of VN, infected the cells with these Ad-uPAR, Ad-p16, and Ad-uPAR/p16 adenovirus vectors, and examined the expression of $\alpha_\text{v}\beta_3$ integrin and the integrin-mediated biological effects.

**Results**

*Expression of p16 and uPAR proteins*

Western blotting analyses confirmed that uPAR protein levels were reduced after glioma cells were infected with the Ad-uPAR or Ad-uPAR/p16 constructs (Figure 1). Expression of uPAR protein in the Ad-p16-infected cells was no different than that in cells that had been mock-infected or infected with an Ad-cytomegalovirus (CMV) construct. Conversely, p16 protein was detected in the Ad-p16- and Ad-uPAR/p16-infected cells but not in the mock-, Ad-CMV-, or Ad-uPAR-infected cells (Figure 1). The $\alpha$-tubulin level did not change under any of the above conditions, indicating that similar amounts of protein had been loaded in each lane.

*Expression of $\alpha_\text{v}\beta_3$ integrin heterodimer*

Next, we used fluorescence-activated cell sorting (FACS) and immunocytochemical analysis with LM609, an antibody specific for hetero-dimeric $\alpha_\text{v}\beta_3$, to assess the cell-surface expression of this integrin. FACS analyses showed that the proportions of $\alpha_\text{v}\beta_3$-positive cells in the Ad-uPAR- (Figure 2e), Ad-p16- (Figure 2f), and Ad-uPAR/p16- (Figure 2g) treated cells were less than those in the mock-infected (Figure 2c) or Ad-CMV-infected (Figure 2d) controls. Similar results were found in immuno-cytochemical
tests (Figure 3); in addition, cells infected with the test constructs were larger and more rounded than the small, spindle-shaped control cells.

Adhesion, and migration.

Next, we assessed the effect of infection with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 on the adhesion of SNB19 cells cultured on VN-coated plates. Adhesion of the Ad-uPAR-infected cells was 43.9% of that in the mock-infected controls; that of Ad-p16-infected cells was 31.0%; and that of Ad-uPAR/p16-infected cells was 29.5% (Figure 4).

Results from a spheroid-migration assay are shown in Figure 5. Mock-infected (Figure 5a) and Ad-CMV-infected (Figure 5b) SNB19 cells were able to migrate from spheroids composed of those cell types. In contrast, spheroids of glioma cells that had been infected with the Ad-uPAR (Figure 5c), Ad-p16 (Figure 5d), or Ad-uPAR/p16 (Figure 5e) constructs showed greatly reduced migration.

Proliferation, survival, and expression of Akt and MAP kinase

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess the effect of the adenoviral vectors on the proliferation of cells cultured on VN-coated micro plates. By 4 days after infection, the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-infected SNB19 cells all showed a decrease in proliferation relative to that of the controls (Figure 6). By 6 days after infection, survival (relative to that of the controls) was only 39.2% in the Ad-uPAR-infected cells, 36.7% in the Ad-p16-infected cells, and 20.9% in the Ad-uPAR/p16-infected cells.
To ascertain whether apoptosis was occurring in the treated SNB19 cells, we used terminal deoxynucleotidyl transferase end labeling and flow cytometry to compare the extent of cell death among the test conditions. On the third day after infection, only 0.13% of the mock-infected cells and 0.23% of the Ad-CMV-treated SNB19 cells were apoptotic. By contrast, at that time 11.0% of the Ad-uPAR-treated cells, 11.5% of the Ad-p16-treated cells, and 17.6% of the Ad-uPAR/p16-treated SNB19 cells were apoptotic (Figure 7).

The phosphatidylinositol 3-kinase- (PI3K)-Akt pathway and mitogen-activated protein kinase (MAPK) cascade are known to regulate signal transduction through integrins and play major roles in cell proliferation and survival. Thus, we used western blotting to compare the total and phosphorylated forms of ERK and Akt among the various test conditions. The amounts of total ERK and Akt proteins expressed by the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-infected cells were slightly lower than those expressed by the mock-infected and Ad-CMV-infected cells, as were the amounts of phosphorylated forms of both proteins (data not shown). Finally, we performed western blotting for Bcl-X\textsubscript{L}, a mitochondrial anti-apoptotic protein whose free form is increased by phosphorylated Akt. Bcl-X\textsubscript{L} expression also was reduced in the Ad-uPAR-, Ad-p16- and Ad-uPAR/p16-infected cells relative to that in the control conditions (Figure 8).

**Discussion**

In this study, we used Ad-uPAR, Ad-p16, and a bicistronic Ad-uPAR/p16 adenovirus vector to infect SNB19 glioma cells cultured in the presence of VN.
blotting showed that SNB19 cells expressed high levels of uPAR protein and that Ad-uPAR and Ad-uPAR/p16 could downregulate its expression. Although SNB19 cells do not normally express the p16 protein, infection with Ad-p16 and Ad-uPAR/p16 resulted in strong expression of this protein. FACS and immuno-cytochemical analyses confirmed that mock-infected and Ad-CMV-infected SNB19 cells expressed high levels of αvβ3 integrin.

On the other hand, cells infected with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 cells clearly showed fewer αvβ3-positive cells and less expression of αvβ3 per cell than the mock-infected or Ad-CMV-infected cells, suggesting that adenovirus-mediated transfer of antisense uPAR and sense p16 gene could downregulate the expression of αvβ3 integrin in glioma cells.

We did all of our experiments under serum-free conditions on VN-coated plates to focus on the reaction between VN and its receptor; serum contains several ECM components and various growth factors, and signaling pathways that are activated by integrin receptors are extensively intertwined with the signaling pathways of growth factors (5, 28). Binding between ECM and integrin receptors leads to the formation of focal adhesion complexes, which play an important role in modulating cell adhesion and inducing changes in cell shape (5). In one study, human embryonic kidney epithelial cells that were made to express αvβ3 adhered to VN-coated plates, but the parental cells, which do not express αvβ3, did not (6). In our study, cells infected with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 showed less adhesion and expressed less αvβ3 than mock- or Ad-CMV-infected cells; moreover, the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-infected
cells also showed changes in shape from small and spindle-shaped to large and rounded. We found that mock infection or Ad-CMV infection did not affect the ability of SNB19 cells to migrate from spheroids, but cells infected with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 showed significantly reduced migratory ability. Another group suggested that activated MAP kinase can promote cell migration through activating myosin light chain (MLC) kinase and then phosphorylating MLC (34). In our experiments, expression of phosphorylated ERK was lower in the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-infected cells than in the controls, a finding that could contribute to the reduced migration of these cells. We previously reported that adenovirus-mediated transfer of the p16 gene suppressed glioma invasion (35). The mechanism underlying this effect was not clear but could be connected to the downregulation of αvβ3 integrin and its downstream cascades by the restoration of p16.

The adhesion-dependant activation of MAP kinase seems to be important in the regulation of cell proliferation by integrins (5). αvβ3 integrin is linked to the Ras-ERK signaling pathway by the adapter protein Shc (36). In our experiments, infection with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 reduced the expression of phosphorylated ERK relative to that of controls. In Brassard et al. ‘s study of human embryonic kidney epithelial cells, cells made to express αvβ3 had greater proliferation on VN-coated plates than did the non-αvβ3-expressing parental cells (6). Others have suggested that the dormancy in human HEp3 carcinoma cells induced by downregulation of uPAR involves integrin and MAP kinase (37). We found that downregulation of uPAR suppressed proliferation in SNB19 cells.
In Brassard et al.’s study of human embryonic kidney epithelial cells, treatment of the cells made to express \( \alpha v \beta 3 \) with \( \alpha v \beta 3 \) antagonists disrupted adhesion to the VN matrix and induced apoptosis (6). We previously found that stably transfecting SNB19 cells with a uPAR antisense construct produced apoptosis of those cells on VN-coated plates under serum-free conditions (38). In the present study, we used terminal deoxynucleotidyl transferase end-labeling and flow cytometry and showed that apoptotic cell death was much higher in the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-treated cells than in the control cells. Cellular adhesion through integrins results in the activation of PI3K independently of signals from serum factors. The lipid products of PI3K provide a protective signal acting through Akt (5). Activated Akt in turn phosphorylates substrates, resulting in a variety of biological effects including suppression of apoptosis (39). The best-known way that activated Akt suppresses apoptosis is by phosphorylating a critical serine residue on Bad, a protein that promotes apoptosis by binding to and blocking the activity of Bcl-X\(_L\), a cell-survival factor. Upon phosphorylation, Bad dissociates from Bcl-X\(_L\), which is then free to resume its activity as a suppressor of apoptosis (40). In our experiments, less phosphorylated Akt and Bcl-X\(_L\) were expressed in the Ad-uPAR-, Ad-p16, and Ad-uPAR/p16-infected cells than in the controls, which would promote apoptosis.

Taken together, our findings suggest that adenovirus-mediated transfer of antisense uPAR and sense p16 gene down-regulated both the expression of integrin \( \alpha v \beta 3 \) and the biological effects that depend on integrin-mediated signaling in glioma cells (Figure 9) (5). These results support the concept that the Ad-uPAR/p16 bicistronic construct may have therapeutic value in malignant gliomas.
Materials and methods

Recombinant adenoviruses

We previously generated the replication-deficient recombinant adenoviruses Ad-uPAR, Ad-p16, and the bicistronic construct Ad-uPAR/p16. The Ad-uPAR construct contains a CMV promoter, a truncated 300-bp antisense message complementary to the 5’ end of the uPAR gene, and bovine growth hormone (BGH) polyadenylation (polyA) signal in a mini-expression cassette, which is inserted into the E1-deleted region of the virus (26). The Ad-p16 construct contains a CMV promoter, wild-type p16 cDNA, and SV40 polyA signal in a mini-expression cassette, which is inserted into the E1-deleted region of the virus (27). The Ad-uPAR/p16 construct has two independent mini-expression cassettes (uPAR antisense and p16 sense) in the E1-deleted region, with the p16 cassette inserted downstream of the uPAR cassette in the opposite orientation. The control virus Ad-CMV has a CMV promoter and BGH polyA signal but no gene insert in the E1-deleted region.

Cell culture and infection conditions

We used the established human glioma cell line SNB19, kindly provided by Dr Richard Morrison, The University of Texas M. D. Anderson Cancer Center, Houston, TX, for this study. Cells were grown in Dulbecco’s modified Eagle medium (DMEM)/F12 medium (1:1, v/v) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37°C.
Tissue culture dishes, 96-well microplates, and chamber slides were coated with VN from human plasma (Sigma, St. Louis, MO) to a surface concentration of 500 ng/cm². For immuno-cytochemical analyses, 100 µl (5µg/ml) of VN diluted in phosphate-buffered saline (PBS) was added to each well of the chamber slides (LabTec/NUNC, Rochester, NY). For the adhesion and proliferation assays, 100 µl (5 µg/ml) of VN diluted in PBS was added to each of the 96 wells in the microplates (Falcon, Franklin Lakes, NJ). For other experiments, 5 ml of VN diluted in PBS (5 µg/ml) was added to 100-mm tissue culture dishes (Corning Inc., Corning, NY). After the VN was added, the dishes, plates, and slides were stored at 4°C overnight, washed with PBS, air-dried, and used immediately.

Cell cultures were maintained in medium containing 10% FBS, but all experiments were performed under serum-free conditions as follows. Viral stocks were suitably diluted in serum-free medium to obtain the desired multiplicity of infection (MOI) or plaque-forming units (PFU), added to cell monolayers prepared in 100-mm plates as described below, and incubated at 37°C for 1 h. The necessary amount of culture medium without serum was then added to the cell cultures, and the cells were incubated for the desired periods.

Fluorescence-activated cell sorting

SNB19 cells (2 x 10⁶) were seeded on VN-coated 100-mm tissue culture plates, incubated for 24 h, and infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16. A mock-infection control condition involved the addition of 10 µl of PBS to the plates. After another 72-h incubation, cells were treated with trypsin/EDTA, washed with PBS,
pelleted at 1000 rpm for 5 min, and re-suspended at a concentration of $1 \times 10^6$ cells/ml in PBS. Cells were then incubated with either control (mouse IgG) antibody (Santa cruz #sc-2025) or LM609, an $\alpha_v\beta_3$ integrin heterodimer-specific monoclonal antibody (Chemicon International, Temecula, CA) (1:250 dilution) for 1 h on ice, pelleted, and washed three times with PBS to remove excess primary antibody. Cells were then resuspended in 1 ml of PBS and incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) (1:250 dilution) for 1 h on ice. After three more washes, streptavidin-FITC conjugates (Gibco BRL, Grand Island, NY) (1:150 dilution) were added, the cells were washed three times again, and the cell pellet was resuspended in 2% paraformaldehyde and analyzed on a Coulter EPICS XL AB6064 flow cytometer (Beckman Coulter, Fullerton, CA).

Immunocytochemical analysis

SNB19 cells ($1 \times 10^4$) were seeded on VN-coated 8-well chamber slides, incubated for 24 h, and infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16. After another 72 h, cells were fixed with 3.7% formaldehyde and incubated with 1% bovine serum albumin in PBS at room temperature for 1 h for blocking. After the slides were washed with PBS, either mouse IgG or LM609 (1:500 dilution) was added and the slides were incubated at room temperature for 1 h and washed three times with PBS to remove excess primary antibody. Cells were then incubated with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) (1:500 dilution) for 1 h at room temperature and then washed three times, after which streptavidin-HRP conjugates were added and the cells incubated for another 45 min. After another three washes, DAB solution (Vector
Laboratories) was added, the slides were covered with glass coverslips, and photomicrographs were obtained.

**Adhesion assay**

Adhesion was assessed as described previously (21) with modifications. SNB19 cells (1 x 10^6) were seeded on VN-coated 100-mm tissue culture plates. After 24 h incubation, cells were infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16 or Ad-uPAR/p16. After another 72 h, cells were harvested by trypsin/EDTA treatment, washed with PBS, resuspended in 10% serum-containing medium, and allowed to recover from the trypsinization for 1 h at 37°C. Cells were washed twice with serum-free medium, resuspended in serum-free medium, and seeded at 2 x 10^4 cells/well in VN-coated 96-well plates. After 2 h incubation at 37°C, unattached cells were removed by rinsing the slides three times with PBS. The remaining attached cells were quantified by measuring the conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT 1mg/ml) (Sigma, St. Louis, MO), to formazan (O.D at 540 nm).

**Migration of cells from spheroids**

Migration was assayed by a previously described method (21, 26) with modifications. Spheroids of SNB19 cells were prepared by suspending 2 x 10^6 cells in DMEM, seeded on 100-mm tissue culture plates coated with 0.75% agar, and cultured until spheroid aggregates formed. Spheroids measuring approximately 150 μm in diameter (about 4 x 10^4 cells/spheroid) were selected and infected with adenovirus vectors at 50 MOI. Three
days after infection, a single glioma spheroid was placed in the center of each well in VN-coated 96-well microplates and 200 µl of serum-free medium was added to each well. Spheroids were cultured at 37°C for 48 h, after which the spheroids were fixed and stained with Hema-3 and migration from the spheroids was assessed under light microscopy.

**Proliferation assay**

Cell proliferation was assessed by seeding 2 x 10³ SNB19 cells in VN-coated 96-well microplates and 24 h later infecting them with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16. At specified times after adenovirus infection, medium was removed from the wells, the cells were rinsed with PBS, and the numbers of viable cells were assessed by the MTT assay.

**Apoptosis assay**

Apoptotic cells were detected by using terminal deoxynucleotidyl transferase end-labeling (APO-BrdU; Phoenix Flow Systems, San Diego, CA) and FACS. Briefly, 1 x 10⁶ SNB19 cells were seeded on VN-coated 100-mm tissue culture plates, incubated for 24 h, and infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16. After another 72 h, cells were harvested, washed in PBS, and fixed in 1.0% paraformaldehyde and ice-cold 70% ethanol. The fixed cells were then washed twice and the cell pellets incubated for 60 min at 37°C in a labeling reaction mixture containing TdT reaction buffer, Br-dUTP, dH₂O, and TdT. The reaction was terminated by the addition of a rinse buffer. Incorporated Br-dUTP was detected after the addition of
fluorescein-labeled anti-BrdU antibody and incubation for 30 min at room temperature in the dark. The amount of DNA in the cells was quantified by adding propidium iodide/RNase A solution and incubating the tubes in the dark for an additional 30 min. After FACS gates were established with intact cells, the cells were analyzed for amount and fragmentation of DNA to determine the percentage of apoptotic cells.

**Immunoblotting**

For these experiments, 2 x 10⁶ SNB19 cells were seeded on VN-coated 100-mm tissue culture plates, incubated for 24 h, and infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16. After another 72 h, cells were lysed as follows. For immunoblotting of all proteins except uPAR, total cell lysates were prepared by the addition of RIPA buffer (150 mM NaCl, 1% NONIDET P-40, 1 mM sodium orthovanadate, and 5 mM EDTA, pH 7.4), aprotinin, and phenylmethylsulfonyl fluoride (PMSF). For immunoblotting of uPAR, an extraction buffer for membrane fractions was used (0.1 M Tris [pH 7.5], 1% Triton-X114, 10 mM EDTA, aprotinin, and PMSF). The extracts were incubated at 37°C for 10 min and centrifuged to separate the lower (detergent) phase, which contains mostly hydrophobic membrane proteins, including the glycosylphosphatidylinositol-anchored uPAR. Twenty micrograms of protein from each sample was subjected to 15% (for p16 or Bcl-X₁), 10% (for uPAR, ERKs, Akts, or α-tubulin) or 7.5% SDS-Tris-glycine gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membranes were probed with the following primary antibodies: rabbit anti-human p16 polyclonal antibody (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-human uPAR polyclonal
antibody (#399R; American Diagnostics Inc., Greenwich, CT), goat anti-ERK1 polyclonal antibody (C-16; Santa Cruz Biotechnology), mouse anti-phospho p44/42 MAP kinase monoclonal antibody (E10; New England Biolabs, Inc., Beverly, MA), rabbit anti-Akt polyclonal antibody (#9272; New England Biolabs), rabbit anti-phospho Akt polyclonal antibody (#9271S; New England Biolabs), and rabbit anti-human Bcl-XL polyclonal antibody (S-18; Santa Cruz Biotechnology). Mouse anti-human α-tublin monoclonal antibody (Ab-1; Calbiochem, San Diego, CA) was used as a loading control. Secondary antibodies (anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase) were used as required, and the membranes were developed according to an enhanced chemiluminescence protocol (Amersham Pharmacia Biotech, UK).

Acknowledgments

This work was supported by NIH grants CA7557, CA76350 (to J.S.R).
References


Cell, 87, 733–743.


Figure 1. Western blot analysis of uPAR and p16 proteins. SNB19 cells were infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16 on VN-coated plates under serum-free conditions. Cells were then lysed and the lysates subjected to SDS-PAGE and immunoblotting with anti-uPAR and anti-p16 antibodies. Anti-α-tubulin antibodies were used to verify that similar amounts of protein had been loaded in each lane.
Figure 2. FACS analysis of αvβ3 integrin heterodimer expression. SNB19 cells were seeded on VN-coated tissue culture plates, infected with 100 MOI of the vectors as indicated below, and 72 h later were harvested, stained with an αvβ3 integrin heterodimer-specific monoclonal antibody, biotinylated anti-mouse IgG, and streptavidin-FITC conjugates, and then analyzed by FACS. (a) Negative control, in which isomatch mouse IgG was used as the primary antibody in uninfected SNB19 cells. (b) SNB19 cells cultured on uncoated plates with medium containing 10% FBS. (c) Mock-infected SNB19 cells. (d) Ad-CMV-infected SNB19 cells. (e) Ad-uPAR-infected SNB19 cells. (f) Ad-p16-infected SNB19 cells. (g) Ad-uPAR/p16-infected SNB19 cells.
Figure 3. Immunocytochemical analysis of αvβ3 integrin heterodimer expression. SNB19 cells were seeded on VN-coated chamber slides and infected with 100 MOI of the vectors as indicated below; 72 h later, the cells were fixed, stained with an αvβ3 integrin heterodimer-specific monoclonal antibody, biotinylated anti-mouse IgG, streptavidin-HRP conjugates and DAB solution. (a) Negative control, in which isomatch mouse IgG was used as the primary antibody instead of anti-αvβ3 integrin heterodimer antibody. (b) Mock-infected SNB19 cells. (c) Ad-CMV-infected SNB19 cells. (d) Ad-uPAR-infected SNB19 cells. (e) Ad-p16-infected SNB19 cells. (f) Ad-uPAR/p16-infected SNB19 cells. Original magnification, 200x.
Figure 4. Adhesion assay. Cells were infected with the various vectors, trypsinized 72 h later, allowed to recover for 1 h in serum-containing medium, washed, resuspended in serum-free medium, and then incubated on VN-coated microplates. Two h later, unattached cells were removed by washing with PBS, and attached cells were quantified by MTT assay. Shown are the mean (± SD) values from five separate experiments.
Figure 5. Migration from spheroids. Glioma-cells spheroids were prepared, infected with 100 MOI of the vectors indicated below, and transferred 72 h later to VN-coated 96-well plates, where they were incubated for 48 h, fixed, and stained as described in Materials and Methods. (a) Mock-infected SNB19 cells. (b) Ad-CMV-infected SNB19 cells. (c) Ad-uPAR-infected SNB19 cells. (d) Ad-p16-infected SNB19 cells. (e) Ad-uPAR/p16-infected SNB19 cells. Original magnification, 100x.
Figure 6. Proliferation assay. Briefly, $2 \times 10^3$ SNB19 cells were seeded in VN-coated 96-well microplates under serum-free conditions and then PBS, Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16 was added. Numbers of viable cells were assessed by the MTT assay. Shown are the mean (± SD) values from five separate experiments.
Figure 7. Apoptosis assay. SNB19 glioma cells were infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16, and 3 days later were fixed and stained for TdT end-labeling and flow cytometric analysis of DNA content. Shown are the mean (± SD) values from three separate experiments.
Figure 8. Western blot analyses of Akt, ERK and Bcl-X<sub>L</sub>. SNB19 cells were infected with 100 MOI of Ad-CMV, Ad-uPAR, Ad-p16, or Ad-uPAR/p16 on VN-coated plates under serum-free conditions. Cells were lysed 72 h later and subjected to SDS-PAGE and immunoblotting with anti-ERK1 antibody, anti-phospho p44/42 MAP kinase antibody, anti-Akt antibody, anti-phospho-Akt antibody, or anti-Bcl-X<sub>L</sub> antibody. Anti-α-tubulin antibodies were used to verify that similar amounts of protein had been loaded in each lane.
Figure 9. Relationship among uPAR, p16, integrin αvβ3, and the integrin-mediated signaling pathways leading to cell adhesion, migration, proliferation, and survival.
Vitronectin expression in vivo: SNB19 cells expressing GFP were injected into nude mice brain. Mice were sacrificed after 4 weeks and vitronectin expression was demonstrated in situ. A&C show nude mice brain sections at 4x and 10x magnification negative control for mouse IgG used as primary antibody. B&D show the vitronectin expression at 4x and 10x magnification (shown in arrows)
Downregulation of integrin αvβ3 expression and integrin-mediated signaling in glioma cells by adenovirus-mediated transfer of antisense uPAR and sense p16 genes
Yoshiaki Adachi, Sajani S. Lakka, Nirmala Chandrasekar, Niranjan Yanamandra, Christopher S. Gondi, Sanjeeva Mohanam, Dzeng H. Dinh, William C. Olivero, Meena Gujrati, Takashi Tamiya, Takashi Ohmoto, Bharat Aggarwal and Jasti S. Rao

J. Biol. Chem. published online September 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104334200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts