Down-regulation of Integrin $\alpha_\beta_3$ Expression and Integrin-mediated Signaling in Glioma Cells by Adenovirus-mediated Transfer of Antisense Urokinase-type Plasminogen Activator Receptor ($uPAR$) and Sense $p16$ Genes*

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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 $\alpha_\beta_3$ 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 $\alpha_\beta_3$ integrin. Moreover, restoration of the p16 protein in glioma cell line SNB19 with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 deleted region of the vector. Infecting the malignant glioma cell with $uPAR$ antisense and $p16$ sense 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 $\alpha_\beta_3$ 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.

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 are 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),1 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 $\alpha_\beta_3$ and $\alpha_\beta_3$ integrins. The $\alpha_\beta_3$ integrin heterodimers in particular are 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 $\alpha_\beta_3$ 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 (7, 9, 10) has used the LM609 anti-$\alpha_\beta_3$ heterodimer antibody to produce tumor regression in vitro and in vivo models. More recently, peptidomimetic inhibitors selective for the $\alpha_\beta_3$ integrin heterodimers have also demonstrated anti-tumor effects in germ cell tumors (11, 12) and gliomas (13).

The urokinase-type plasminogen activator receptor ($uPAR$) is a single-chain, highly glycosylated protein with a molecular weight of 50,000–60,000 that is anchored on the cell membrane by a glycosylphosphatidylinositol moiety (14). Urokinase-type plasminogen activator 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 $\alpha_\beta_3$ mRNA (18). Our stud-

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‡‡‡ The abbreviations used are: VN, vitronectin; ECM, extracellular matrix; uPAR, urokinase-type plasminogen activator receptor; CMV, cytomegalovirus; PBS, phosphate-buffered saline; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAB, 3,3'-diaminobenzidine; FACS, fluorescence-activated cell sorting; TdT, terminal deoxynucleotidyl transferase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein.
ies and others showed that uPAR levels are significantly increased during the progression of human gliomas (19, 20) and tumor formation and tumor growth is inhibited in antisense uPAR clones (21, 22).

In gliomas, the p16 tumor suppressor gene is frequently inactivated (23, 24). The p16 protein, molecular weight 16,000, acts as a cyclin-dependent kinase inhibitor, inhibiting the binding of the Cdk4 and Cdk6 proteins to cyclin D1. Recent reports indicate that restoration of p16 protein inhibited down-regulation of the Cdk4 and Cdk6 proteins to cyclin D1. Recent reports inactivated (23, 24). The p16 protein, molecular weight 16,000, does not encode a CMV promoter and bone marrow polyadenylation (poly(A)) signal to the 5′-end of the uPAR gene, and bovine growth hormone polyadenylation (poly(A)) 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, a truncated 300-base pair antisense message complementary to the 5′-end of the uPAR gene, and bovine growth hormone polyadenylation (poly(A)) 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 bovine growth hormone poly(A) 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 (University of Texas M. D. Anderson Cancer Center, Houston, TX), for this study. Cells were grown in Dulbecco’s modified Eagle medium/F12 medium (1:1, v/v) supplemented with 10% fetal bovine serum 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) to a surface concentration of 500 ng/cm2. For immunocytochemical analyses, 100 µl (5 µg/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, Becton Dickinson, Lakes, NJ). For other experiments, 5 µl of VN diluted in PBS (5 µg/ml) was added to 100-mm tissue culture dishes (Corning Inc.). 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% fetal bovine serum, 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, 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 × 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 at 60% confluence. Cells were then infected with either control (mouse IgG) antibody (Santa Cruz, sc-2025) or LM609, an αβ3 integrin heterodimer-specific monoclonal antibody (Chemicon Int., Temecula, CA), in serum-free medium (1:100 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-fluorescein isothiocyanate conjugates (Life Technologies,
Ad-p16-infected SNB19 cells.

Immunocytochemical Analysis—SNB19 cells (1 x 10^6) 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; 1:500 dilution) for 1 h at room temperature for 1 h and washed three times with, after which streptavidin-horseradish peroxidase conjugates, and 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 a 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, and fixed with 3.7% 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, bromo-dUTP, DHzO, and TdT. The reaction was terminated by the addition of a rinse buffer. Incorporated bromo-dUTP was detected after the addition of fluorescein-labeled anti-deoxybromouridine 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 the amount and fragmentation of DNA to determine the percentage of apoptotic cells.

Immunoblotting—For these experiments, 2 x 10^6 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 radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 5 mM EDTA, pH 7.4),
aprotinin, and phenylmethylsulfonyl fluoride. 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 phenylmethylsulfonyl fluoride). 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. 20 μg of protein from each sample was subjected to 15% (for p16 or Bcl-XL) or 10% (for uPAR, ERKs, Akts, or α-tubulin) SDS-Tris-glycine gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc.). 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 (E10; New England Biolabs, Inc., Beverly, MA), rabbit anti-Akt polyclonal antibody (no. 9272; New England Biolabs), and rabbit anti-human Bcl-XL polyclonal antibody (S-18; Santa Cruz Biotechnology). Mouse anti-human α-tubulin 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).

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 (Fig. 1). Expression of uPAR protein in the Ad-p16-infected cells was no different from 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 (Fig. 1). The α-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 αvβ3 Integrin Heterodimer—Next, we used fluorescence-activated cell sorting (FACS) and immunocytochemical analysis with LM609, an antibody specific for heterodimeric αvβ3, to assess the cell-surface expression of this integrin. FACS analysis showed that the proportions of αvβ3-positive cells in the Ad-uPAR (Fig. 2E), Ad-p16 (Fig. 2F), and Ad-uPAR/p16 (Fig. 2G)-treated cells were less than those in the mock-infected (Fig. 2C) or Ad-CMV-infected (Fig. 2D) controls. Similar results were found in immunocytochemical tests (Fig. 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% (Fig. 4).

Results from a spheroid migration assay are shown in Fig. 5. Mock-infected (Fig. 5A) and Ad-CMV-infected (Fig. 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 (Fig. 5C), Ad-p16 (Fig. 5D), or Ad-uPAR/p16 (Fig. 5E) constructs showed greatly reduced migration.

**Proliferation, Survival, and Expression of Akt and MAP Kinase**—We used the 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 (Fig. 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 TdT 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 (Fig. 7).

The phosphatidylinositol 3-kinase (PI3K)-Akt pathway and mitogen-activated protein (MAP) kinase cascade are known to regulate signal transduction through integrins and to 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-XL, a mitochondrial anti-apoptotic protein in which the free form is increased by phosphorylated Akt. Bcl-XL expression also was reduced in the Ad-uPAR-, Ad-p16-, and Ad-uPAR/p16-infected cells relative to that in control conditions (Fig. 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. Western blotting showed that SNB19 cells expressed high levels of uPAR protein and that Ad-uPAR and Ad-uPAR/p16 could down-regulate its expression. Although SNB19 cell do not normally express the p16 protein, infection with Ad-p16 and Ad-uPAR/p16 resulted in strong expression of this protein. FACS and immunocytochem-
ical analyses confirmed that mock-infected and Ad-CMV-infected SNB19 cells expressed high levels of $\alpha_\beta_3$ integrin.

On the other hand, cells infected with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 cells clearly showed fewer $\alpha_\beta_3$-positive cells and less expression of $\alpha_\beta_3$-cell than the mock-infected or Ad-CMV-infected cells, suggesting that adenovirus-mediated transfer of the antisense uPAR and sense p16 gene could down-regulate the expression of $\alpha_\beta_3$ 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 $\alpha_\beta_3$ adhered to VN-coated plates, but the parental cells, which do not express $\alpha_\beta_3$, did not (6). In our study, cells infected with Ad-uPAR, Ad-p16, or Ad-uPAR/p16 showed less adhesion and expressed less $\alpha_\beta_3$ 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 kinases promote cell migration by phosphorylation, thereby enhancing the myosin light chain kinase, which in turn leads to the phosphorylation of myosin light chains (29). 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 (30). The mechanism underlying this effect was not clear but could be connected to the down-regulation of $\alpha_\beta_3$ integrin and its downstream cascades by the restoration of p16.

The adhesion-dependent activation of MAP kinase seems to be important in the regulation of cell proliferation by integrins (5). $\alpha_\beta_3$ integrin is linked to the Ras-ERK signaling pathway by the adapter protein Shc (31). 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 the Brassard et al. (6) study of human embryonic kidney epithelial cells, cells made to express $\alpha_\beta_3$ had greater proliferation on VN-coated plates than did the non-$\alpha_\beta_3$-expressing parental cells. Others have suggested that the dormancy in human HEP3 carcinoma cells induced by down-regulation of uPAR involves integrin and MAP kinase signaling (32). We found that down-regulation of uPAR-suppressed proliferation in SNB19 cells. In the study of human embryonic kidney epithelial cells done by Brassard et al. (6), treatment of the cells made to express $\alpha_\beta_3$ with $\alpha_\beta_3$ antagonists disrupted adhesion to the VN matrix and induced apoptosis. 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 (33). In the present study, we used TdT 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 (34). 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-XL, a cell survival factor. Upon phosphorylation, Bad dissociates from Bcl-XL, which is then free to resume its activity as a suppressor of apoptosis (35). In our experiments, less phosphorylated Akt and Bcl-XL 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 the antisense uPAR and sense p16 genes down-regulated both the expression of integrin αβ3 and the biological effects that depend on integrin-mediated signaling in glioma cells (Fig. 9) (5). These results support the concept that the Ad-uPAR/p16 bicistronic construct may have therapeutic value in malignant gliomas.

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