Astrocytoma Adhesion to Extracellular Matrix: Functional Significance of Integrin and Focal Adhesion Kinase Expression

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Abstract. Evidence is accumulating implicating a role for integrins in the pathogenesis of cancer, a disease in which alterations in cellular growth, differentiation, and adhesive characteristics are defining features. In the present report we studied a panel of 8 human astrocytoma cell lines for their expression of integrin subunits by RT-PCR, and of integrin heterodimers by immunoprecipitation analyses. The functionality of integrin heterodimers was assessed using cell attachment assays to plastic or single matrix substrates. Downstream effects of integrin activation were studied by western blot analyses of FAK expression in human astrocytoma cell lines growing on plastic and on a fibronectin matrix, and in 13 primary human brain tumor specimens of varying histopathological grade. Furthermore, we studied tyrosine phosphorylation of FAK in astrocytoma cells growing on plastic versus fibronectin. Finally, we analyzed the effects of intermediate filament gene transfer on FAK phosphorylation in SF-126 astrocytoma cells. Our data show that astrocytoma cell lines express various integrin subunits by RT-PCR, and heterodimers by immunoprecipitation analyses. The β1 and αν integrin subunits were expressed by all astrocytoma cell lines. The α3 subunit was expressed by all cell lines except SF-188. By immunoprecipitation, the fibronectin receptor (α5β1 integrin heterodimer) and the vitronectin receptor (ανβ3) were identified in several cell lines. Astrocytoma cell attachment studies to human matrix proteins suggested that these integrin heterodimers were functional. Using confocal laser microscopy, we showed that FAK was colocalized to actin stress fibers at sites of focal adhesion complexes. By western blot, FAK was variably but quite ubiquitously expressed in human astrocytoma cell lines, and in several primary human astrocytoma specimens. When U373 and U87 MG astrocytoma cells bind to a fibronectin matrix, FAK is phosphorylated. GFAP-transfected SF-126 human astrocytoma cells were shown to overexpress the phosphorylated form of FAK only when these cells were placed on a fibronectin matrix. This result is of interest because it suggests that manipulations of the astrocytoma cytoskeleton per se can bring about potential signaling changes that channel through integrins and focal adhesion sites leading to activation of key kinases such as FAK.

Key Words: Adhesion; Astrocytoma, Cytoskeleton; Focal adhesion kinase; Integrins, Signaling.

INTRODUCTION

Astrocytomas are the most common primary human brain tumors and, in their malignant form, have a very poor prognosis. These tumors are highly proliferative, and they infiltrate diffusely into regions of normal brain rendering total surgical extirpation impossible and effective local radiation therapy difficult. Conventional chemotherapy frequently fails either because most agents administered systemically do not cross the blood-brain barrier, or because of intrinsic tumor cell resistance. Of the hallmark histopathological features of malignant astrocytomas, the invasion of normal brain by astrocytoma cells perhaps explains best why these tumors are frequently recalcitrant to therapy.

The coordination of the steps involved in the invasion of normal tissue by tumor cells requires the regulation of a number of complex cytological processes which include the binding of specific cell-surface molecules on the plasma membrane to the extracellular matrix (ECM), the elaboration of targeted proteases to degrade ECM components, and the interactions between the ECM, plasma membrane and the cytoskeleton, all of which may have profound effects on tumor cell morphology and motility (1–5). We have already studied the process of astrocytoma invasion insofar as the expression of metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs)(6–9) and the effects of glial fibrillary acidic protein (*GFAP*) gene-transfection are concerned (10–12). In the present report, we focused specifically on the properties of astrocytoma cell binding to ECM through an analysis of integrin expression.

The integrins are a family of type I transmembrane proteins composed of a large extracellular domain, a hydrophobic membrane spanning segment, and a short cytoplasmic domain (2, 4, 13). Each integrin is composed of an α and a β subunit. At present, there are at least 15 different α and 9 β subunits that can variously combine to form 21 receptors with distinct ligand specificities (14). Integrins recognize specific peptide regions within ECM macromolecules; the best characterized of which is the arginine-glycine-aspartic (RGD) sequence of fibronectin (2, 13).

Upon binding to extracellular ligands, integrins cluster on the plane of the plasma membrane and promote the assembly of molecular complexes containing both cytoskeletal and signaling elements (14). The effects that the extracellular matrix has on cellular growth and differentiation are likely mediated through various integrin signaling pathways (so called "out:in" signaling) (15). Although the integrin signalling pathways have not been completely characterized, many involve tyrosine kinase phosphorylation of neighbouring protein species and cyclin dependent-kinases (16–21). As one example, the study of ECM-induced aggregation of integrin receptors at focal adhesion sites led to the identification of a phosphorylated 125 kDa protein known as focal adhesion kinase (FAK) (17, 18). FAK has been shown to physically associate with 2 nonreceptor protein tyrosine kinases, pp60src and pp59fyn, via their Src homology 2 (SH2)-domains (18, 20, 21).

In the present report, we have examined a panel of astrocytoma cell lines and primary human astrocytoma specimens for their expression of integrins and FAK. Our data show that astrocytoma cells express a variety of integrins that are functionally active in cell attachment assays. Furthermore, we show that binding of astrocytoma cells to ECM leads to activation of FAK. Finally, modulation of intermediate filament expression in astrocytoma cells by GFAP-transfection leads to marked changes in astrocytoma morphology, adhesion, and integrin repertoire expression. These changes are accompanied by increases in tyrosine phosphorylation of FAK suggesting that in addition to the well-recognized "out:in" signaling pathway mediated by ECM-integrin binding, an "in:out" signalling pathway through integrins may be activated by specific cytoskeletal manipulations.

MATERIALS AND METHODS

Astrocytoma Specimens and Cell Lines, Cell Cultures, and Culture Conditions

Human malignant astrocytoma cell lines SF126, SF188, SF539 have been previously described (22, 23). U87 MG, U138 MG, U251 MG, and U373 MG were obtained from the American Type Culture Collection (Rockville, MD). U343-MG-A and XF498 were obtained from Dolores Dougherty (Brain Tumor Research Center, University of California at San Francisco). Two astrocytoma cell lines in which glial filament expression was modulated by GFAP gene transfection were also used. In the first, the GFAP-negative SF-126 astrocytoma cell line was transfected with an expression vector carrying the GFAP gene in the sense orientation creating GFAP-positive astrocytoma cells (10); in the second, an anti-sense GFAP construct was used to render GFAP-positive U251 MG astrocytoma cells GFAP-negative (11). These 2 cell lines and their respective controls were also utilized in the experiments described below. Cell lines were maintained in alpha minimal essential media (αMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 100 U/mL penicillin, 100 μg/mL

streptomycin and 25 μg/mL amphotericin B (Gibco BRL). XF498 was maintained in RPMI media (Gibco BRL) with identical serum and antibiotic concentrations. Early passage cultures of normal human fibroblasts and human cerebral endothelial cells (24) were used as controls, and were maintained in αMEM as above.

Brain tumor specimens were obtained at the time of craniotomy. These tumors were either low-grade astrocytomas taken from a pediatric population (WHO classification, grade I), or high-grade astrocytomas (WHO grade IV). Specimens of human brain were also obtained during the course of routine craniotomy for arteriovenous malformation, trauma, or epilepsy. Permission to utilize this tissue was given by the Research Ethics Board, The Hospital for Sick Children. Specimens were snap frozen and stored in liquid nitrogen until further use.

Reverse Transcription-PCR Analysis of Integrins

Total RNA was isolated from monolayer cultures according to the methods of Chomczynski using RNAzol® (25). Five μg of total RNA, 200 pmol of 3' β-actin primer and 200 pmol of 3' integrin subunit primer were ethanol precipitated and redissolved in 12.5 μl of DEPC-treated H20. The samples were heated at 65°C for 10 minutes (min) and left at room temperature for 2 min. Reverse transcription (RT) synthesis of complementary DNA (cDNA) was performed according to the manufacturer's recommendations (Invitrogen, San Diego, CA). Primer pairs for each of the integrin subunits utilized were derived from previously published material, and are listed below (26–28):

Integrin β1[28]   5' AAT GGG AAC AAC GAG GTC ATG GTT 3'
                 5' TTG TGG GAT TTG CAC GGG CAG TAC 3'

Integrin β3[28]   5' TGC TCA TTG GCC TTG CCG CCC TGC 3'
                 5' TGA TCT GAG GAT GAC TGC TTA TCA 3'

Integrin α2[27]   5' TGG GGT GCA AAC AGA CAA GG 3'
                 5' GTA GGT CTG CTG GTT CAG C 3'

Integrin α3[26]   5' TGG GCA GAT GGA TGT GGA TGA GAA 3'
                 5' GAT GAT GAT GGG GCG GAG TTT GTC 3'

Integrin α5[27]   5' GCG CTC CAC TGT ACA GCT G 3'
                 5' CAG CAA GTC ATC CAG CCC G 3'

Integrin αv[27]   5' GAG CAG CAA GGA CTT TGG G 3'
                 5' GGG TAC ACT TCA AGA CCA GC 3'

For amplification of DNA, 5 μl of the RT product was added to 31.5 μl H20, 5 μl 10X PCR buffer, 0.5 μl of 100 mM dNTPs, and 2 μl of each 5' primer (β-actin and integrin subunit primer), and 1.5 μl of each 3' primer. Then, 1 μl of Taq polymerase was added immediately prior to use, bringing the total volume of the reaction to 50 μl. Samples were covered with mineral oil and placed in a thermal cycler (Perkin-Elmer, Cetus). Thermal cycling was initiated with a 2 min "hot start" at 95°C. The denaturation step was set for 1 min at 95°C, annealing for 1 min at varied temperatures (see below), and extension for 1 min at 75°C. To reduce nonspecific primer annealing, a "touchdown" protocol was employed by setting the initial primer annealing temperature above normal. Thus, the annealing temperature was set for 68°C for the first 5 cycles, followed by 5

TABLE 1
Integrin Subunit Expression of mRNA in Human Astrocytoma Cell Lines

Integrin/ actin ratio*	U87 MG	SF-126	SF-188	U-251	U343	XF-498	SF-539	Fibroblasts
β1/actin	+++	+	+++	++	++	+	++	++
β3/actin	+++	0	+	+	0	0	+	+
α2/actin	++	+	0	+	0	0	0	0
α3/actin	+++	++	0	++	+++	+	++	0
α5/actin	+	+	+	+	0	0	+	0
αv/actin	+	+	+	+	+	+	++	++

* + = ratio of integrin subunit/actin between 0.25–0.33 by RT-PCR. ++ = ratio between 0.5–0.67. +++ = ratio between −0.75–1.0. 0 = no integrin band visualized.

TABLE 2
Integrin Subunit Expression of mRNA in Antisense-GFAP Transfected U251 MG Astrocytoma Cells

Integrin/actin ratio	asGFAP**	asGFAP control~	Parental U251
β1/actin	+++*	+	++
β3/actin	+++	+	+
α3/actin	++	++	++
α5/actin	+	+	+
αv/actin	+	+	+

* + = ratio of integrin subunit/actin between 0.25–0.33 by RT-PCR. ++ = ratio between 0.5–0.67. +++ = ratio between −0.75–1.0. 0 = no integrin band visualized.
** Stable U251 clone #6 transfected with expression vector containing antisense GFAP cDNA.
~ Stable U251 clone transfected with expression vector minus antisense GFAP cDNA.

cycles at 66°C, 10 cycles at 64°C, and 10 cycles at 60°C. Samples of 10 μl were taken at 20, 25, and 30 cycles, and run in 1.6% agarose gels. The gels were stained with ethidium bromide and photographed. Densitometry was performed on the film negative. Integrin expression was calculated as the ratio between the integrin and the β-actin bands.

Astrocytoma Cell Attachment Assay

Nontissue culture microtiter wells were coated with 5–10 μg/ml concentrations of human fibronectin, type IV collagen, laminin, and vitronectin (Telios Pharmaceuticals, San Diego, CA). Before the addition of 3×10^4 astrocytoma cells or human cerebral capillary endothelial cells, unbound sites were blocked with 1% BSA (Sigma) in DMEM for 1 hour (h) at 37°C. The cells were plated in triplicate wells in 1% BSA-containing DMEM. The attached cells were incubated for 30 min (at 37°C, 5% CO_2) washed in PBS to remove floating cells, and fixed in 3.7% paraformaldehyde and stained with 0.5% toluidine blue. For blocking experiments, astrocytoma cells were incubated with neutralizing anti-integrin antibodies or mouse IgG (50 μg/ml) for 30 min before aliquots were placed into the wells. Cell attachment was quantitated by measuring absorbance in each well at 570 nm in a microtiter plate reader as described previously (29). The results of all experiments were expressed as a percentage of cell attachment on substrate referable to astrocytoma cells attaching to plastic alone. Each attachment assay

was repeated twice. The results represent the mean attachment of cells to substrate based on 2 repeats of triplicate well data points.

Antibodies and Reagents

Polyclonal antibodies to FAK (C-20) and to tyrosine phosphorylated proteins for Western blotting (PY20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Integrin subunit antibodies β1(30), β3 (31), β4 (32) α2 (33), α3 (33), α4 (34), α5 (30) and αvβ5 were purchased from Becton Dickinson (San Jose, CA); antibodies to α6 (35) were from CLB (Amsterdam, Netherlands); and antibodies to αv (36) were from Calbiochem (San Diego, CA); an α5β1 antibody was purchased from Gibco BRL (Grand Island, NY). Antibodies to αvβ3 were the generous gift of Dr. Shoukat Dedhar, Toronto, Canada. Protein A-Sepharose was purchased from Pharmacia Biotechnology Co (Uppsala, Sweden).

Immunoprecipitation of Integrin Heterodimers

Cells were harvested by scraping and washed in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Membrane proteins were biotinylated by incubating cells in wash buffer containing 50 μg/ml Biotin-NHS-Sulpho (Pierce Chemical Company, Rockford, IL) for 30 min at 37°C with gentle rotation. Cells were washed with PBS prior to lysis in RIPA buffer (1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate in PBS) containing 10 mM PMSF (Sigma). Protein concentration was determined using a modified Bradford Assay (BioRad, Hercules, CA) with BSA standards.

Antibodies to αvβ3 and α5β1 integrins were incubated separately with 100–150 μg of the soluble protein fraction for 1 h at 4°C with gentle rocking. Protein-antibody complexes were incubated with Protein-A sepharose beads for 1 h at 4°C with gentle rocking. Sepharose beads were washed twice in RIPA buffer containing 0.5M NaCl and twice in RIPA buffer only. Pellets were resuspended in 2X sample buffer (0.125M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue) and boiled for 2 min.

Proteins were separated on a 7.5% acrylamide gel along with biotinylated protein markers (BioRad) and transferred using a BioRad semidry transfer apparatus onto PVDF membrane (Millipore, Bedford, MA). The membrane was incubated for 1 h at room temperature in TBST buffer (20mM Tris-HCl, 500mM NaCl, 0.1% Tween 20) containing 1 μg/ml horseradish peroxidase-conjugated streptavidin (Pierce Chemical Company). The
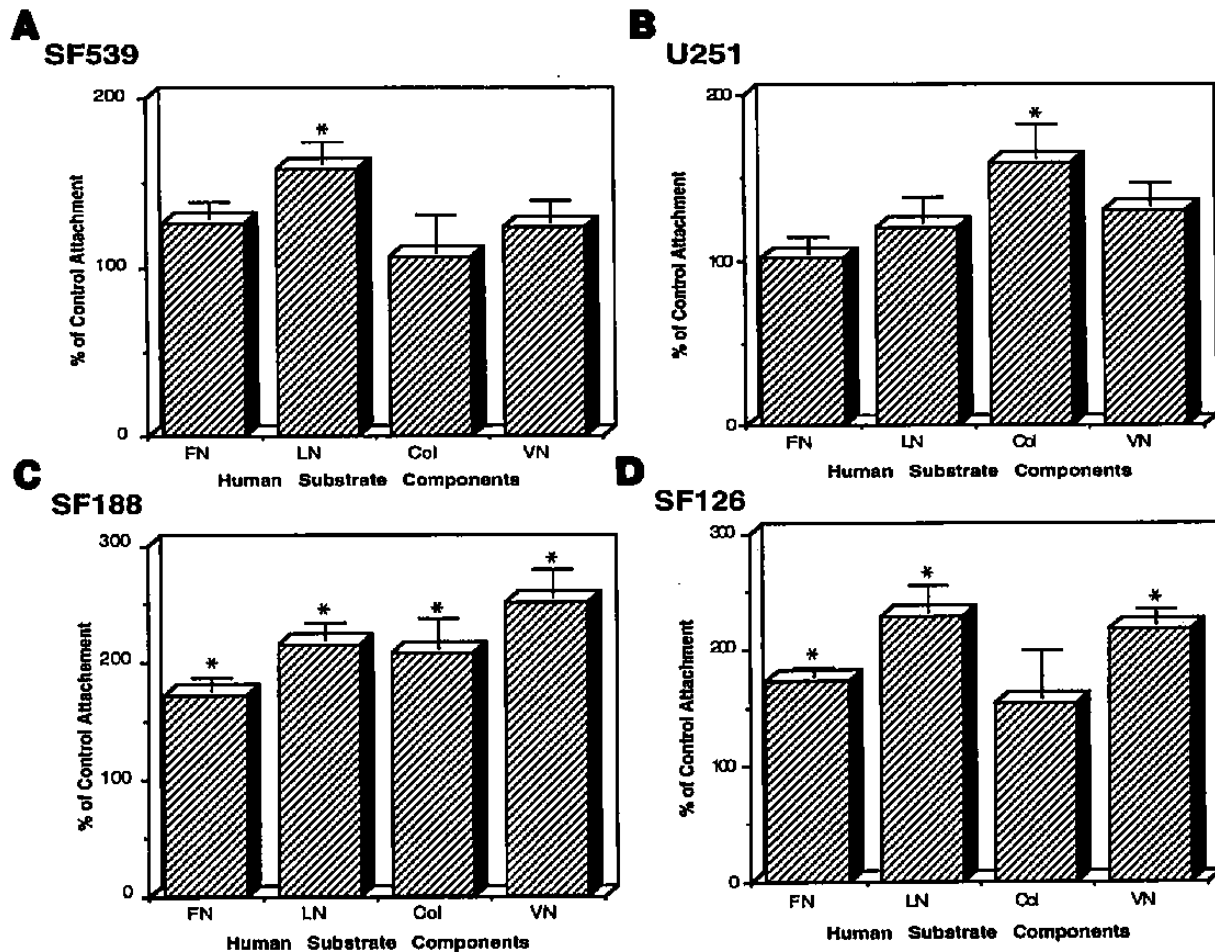
Fig. 1.   Astrocytoma cell attachment to ECM substrates. Control represents astrocytoma cell attachment to plastic alone. A) SF-539 astrocytoma cells show preferential attachment to laminin as a substrate. B) U251 MG astrocytoma cells preferentially attach to type IV collagen. C) SF-188 astrocytoma cells demonstrate multiple ECM ligand affinities including fibronectin, laminin, type IV collagen, and vitronectin. D) SF-126 astrocytoma cells demonstrate multiple ECM ligand affinities including fibronectin, laminin, type IV collagen and vitronectin. Error bars represent standard deviations for cell attachment to ECM substrates. * = Statistically significant differences between cell attachment to specific ECM substrate vs attachment to plastic alone (p < 0.05).

membrane was washed 5–10 times in TBST buffer before incubation for 1 min in ECL chemicals (Amersham Life Science, Arlington Heights, IL). The membrane was then exposed to Kodak XAR film and the film developed.

SDS-PAGE and Western Blot Analysis for FAK

Primary human astrocytoma specimens were homogenized in lysis buffer containing 20 mM Tris-HCl, 138 mM NaCl, 10% glycerol, 1% NP40, 1 mM PMSF, 10 μg/ ml aprotinin, 10 μg/ ml leupeptin, 1 mM sodium orthovanadate, 2 mM EDTA, and 10 mM NaF. Astrocytoma cells in culture were incubated in medium containing 1% BSA for 30 minutes. Astrocytoma cultures were then incubated on ice for 10 min in lysis buffer, scraped, and homogenized. The brain tumor specimens and cell cultures were then centrifuged at 10,000 g for 15 min. Protein samples were electrophoresed on 10% SDS gels containing 0.8% bis-acrylamide according to Laemmli (37) and transferred to PVDF membranes (Millipore). After blocking nonspecific binding, filters were incubated with the FAK primary antibody

for 1 h at room temperature. The filters were then washed and placed in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Visualization of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Amersham Life Science) by exposing the membranes to Kodak XAR film.

Immunoprecipitation and Western Blot Analysis of Tyrosine Phosphorylated FAK

Analysis of tyrosine phosphorylation of FAK was accomplished by immunoprecipitation of FAK followed by Western blot analysis with the anti-phosphotyrosine antibody PY20. First, astrocytoma cultures were incubated in 1% BSA-containing medium for 30 minutes. Cell lysates were then prepared as described above, quantitated, equalized and incubated with antibodies to FAK (Santa Cruz Biotechnology) for 1 h at 4°C with gentle rocking. Protein A sepharose beads (Pharmacia) were added to the lysates containing the protein-antibody complexes and the mixture was incubated at 4°C for an additional hour.

The beads were washed in RIPA buffer 3–5 times before an equal amount of 2X sample buffer containing β-mercaptoethanol was added. The samples were boiled for 2 min and electrophoresed on a 10% acrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore) using the BioRad semidry transfer apparatus. The membrane was blocked in PBS-T containing 5% skim milk powder for 1 h at room temperature before the addition of PY20 antibody. The membrane was washed and an appropriate horseradish peroxidase conjugated secondary antibody was applied. The proteins were visualized by exposure to Kodak XAR film following the enhanced chemiluminescence reaction.

Immunocytochemistry for FAK

For immunofluorescence microscopy of FAK, cells were plated on fibronectin-coated chamber slides, washed and permeabilized for 2 min in 0.02% Triton X-100 before fixing (4% paraformaldehyde in phosphate buffer) for 30 min. After extensive washes the cells were blocked in 0.5% BSA for a total of 15 min. The cells were incubated at room temperature with anti-FAK for 1 h, washed in PBS, and stained with rhodamine-labeled, affinity-purified goat anti-rabbit IgG (H+L) secondary antibody (Pierce). Cells were viewed and photographed on a Leica epiflourescent microscope. In some instances, cells on coverslips were immunostained with FAK followed by a goat anti-rabbit IgG conjugated to FITC (Molecular Probes, OR). They were then rinsed thoroughly with PBS/BSA and treated with Rhodamine-Phalloidin (Molecular Probes). Following a thorough rinse with PBS, the cells were mounted on slides with Elvanol and images obtained simultaneously of both FAK and filamentous actin using a confocal scanning laser microscope (Leica). Images were viewed as a stacked perspective.

RESULTS

Astrocytoma Cell Lines Demonstrate Variable Integrin Expression by RT-PCR

Six integrin subunits were examined (β1, β3, α2, α3, α5, and αv) in all astrocytoma cell lines and human fibroblasts by RT-PCR. These results are shown in Table 1, and represent a ratio between integrin subunit and β-actin band intensities. All cell lines were found to express the β1 and the αv subunits. There was preferential expression of the β1 integrin subunit in U87 MG, and SF-188 astrocytoma cell lines compared to fibroblasts and other astrocytoma cell lines. Only the U87 MG astrocytoma cell line showed strong expression of the β3 integrin subunit. The α2 integrin was expressed only by U87 MG, SF-126, and U251 MG. The α3 integrin was expressed by all astrocytoma cell lines with the exception of SF-188. Of all the astrocytoma cell lines, U87 MG had the greatest expression of the largest number of integrin subunits.

The results of antisense-GFAP transfection on integrin subunit expression in the GFAP-positive U251 MG astrocytoma cell line are shown in Table 2. Elimination of GFAP expression in U251 MG astrocytoma cells led to

TABLE 3
Effects of Anti-integrin Antibodies on SF-188 Cell Adhesion to Single ECM Components

| Anti-integrin Ab | Purified ECM component | | | |
	Collagen type IV	Fibronectin	Laminin	Vitronectin
α2	75 ± 6*	98 ± 7	72 ± 7	94 ± 7
α3	72 ± 8	80 ± 6	83 ± 6	97 ± 6
α4	94 ± 5	85 ± 5	91 ± 8	58 ± 7
α5	98 ± 4	53 ± 7	95 ± 5	92 ± 6
α6	92 ± 6	92 ± 9	62 ± 7	96 ± 8
β1	30 ± 8	64 ± 5	61 ± 8	52 ± 9
β3	60 ± 9	72 ± 8	68 ± 9	61 ± 3
β4	97 ± 7	96 ± 6	59 ± 7	93 ± 7
αv	63 ± 5	72 ± 5	68 ± 6	62 ± 7
αvβ5	51 ± 8	25 ± 4	61 ± 8	60 ± 8

* Numbers indicate percent inhibition of adhesion to purified ECM component (± standard deviation) when measured against control (attachment to substrate in presence of irrelevant antibody, mouse IgG).
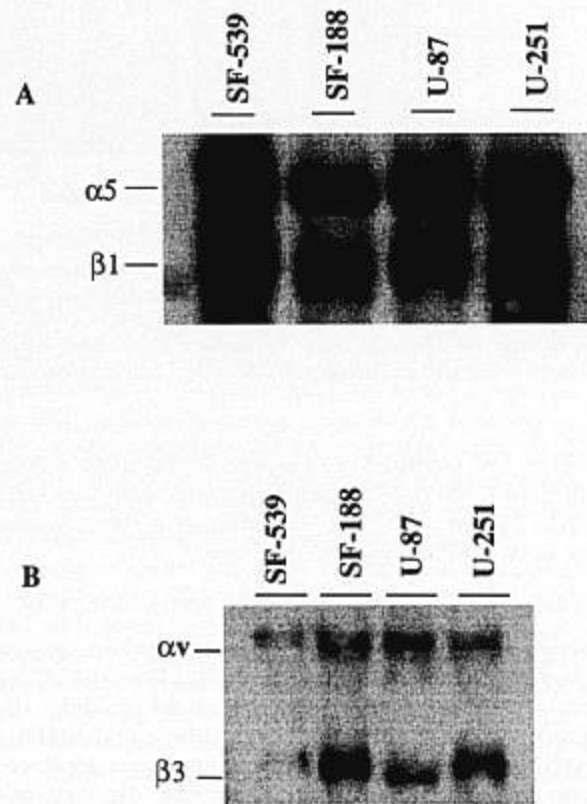

Fig. 2. Immunoprecipitation analysis of integrin heterodimers from astrocytoma cells. A) The fibronectin receptor α5β1 is recognized in lysates from all astrocytoma cell lines using anti-α5β1 antibody (Gibco, BRL). B) The vitronectin receptor αvβ3 is recognized in lysates from all astrocytoma cell lines with variable expression in each cell line (anti-αvβ3 antibody provided by Dr. Shoukat Dedhar).

an increase in expression of the β1 and β3 integrins in the antisense-GFAP transfected astrocytoma cell lines.

Astrocytoma Cell Lines Have Different Affinities for ECM Substrates

The cell attachment assays revealed that the astrocytoma cell lines studied have different affinities for ECM macromolecules as substrates. Some cell lines such as SF-539 and U251 MG showed preferential attachment to single matrix macromolecules, laminin and type IV collagen respectively (Fig. 1A, B). Other astrocytoma cell lines were quite diverse in their ability to attach to multiple ECM components. For example, both SF-188 and SF-126 were capable of attaching readily to vitronectin, type IV collagen, laminin and fibronectin (Fig. 1C, D).

The inhibitory effects of anti-integrin antibodies on the adhesion of SF-188 astrocytoma cells to all purified ECM components was strong for β1, β3, αv, and αvβ5. In addition, adhesion to fibronectin was strongly inhibited by blocking antibodies to α5; adhesion to laminin was strongly inhibited by antibodies to α6; and adhesion to vitronectin was strongly inhibited by antibodies to α4 (Table 3).

Identification of Integrin Heterodimers in Astrocytomas by Immunoprecipitation

By immunoprecipitation, we were able to demonstrate that integrin heterodimers are represented as intact complexes in human astrocytoma cells. Representative blots are found in Figure 2 for the fibronectin (α5β1) and vitronectin (αvβ3) receptors for 4 astrocytoma cell lines. The position of the individual integrin bands identified by immunoprecipitation analysis was confirmed by western blot analysis with integrin specific antibodies (data not shown). The data from our immunoprecipitation experiments, which demonstrated intact integrin heterodimers, were supportive of the cell attachment data for most astrocytoma cell lines suggesting that these heterodimers are functional in their abilities to recognize matrix macromolecules as substrates. These data also were supportive of those obtained by RT-PCR for integrin subunit expression in the different astrocytoma cell lines.

Astrocytoma Cell Lines and Specimens Express FAK

FAK was expressed by western blot analysis by all human astrocytoma cell lines examined. However, expression was variable, with SF-188 expressing the least, and U 373 MG expressing the most compared to human fibroblasts (Fig. 3). As for the primary human astrocytoma specimens examined for FAK expression, the tumors were divided into low-grade and high-grade tumor types. Two of 6 pediatric low-grade (Grade I) astrocytomas expressed FAK at levels above the basal level expressed in nontumorous brain tissue. However, 6 of 7

high-grade (Grade IV) astrocytomas showed expression levels greater than that of normal human brain (Fig. 4).

To determine if FAK levels would be altered on a fibronectin matrix, a western analysis for FAK was performed on astrocytoma cell lines growing on either plastic or fibronectin (Fig. 5). While there were some changes in levels of expression of FAK for some cell lines (e.g. U87 MG grown on fibronectin versus plastic), these changes were not striking. For this reason, attempts were made to study the expression of the phosphorylated form of FAK under similar conditions (see next section). Western blot analyses were performed in all instances for GAPDH to ensure equivalence of protein loading (data not shown).

FAK is Phosphorylated in Astrocytoma Cell Lines and GFAP-Transfectants

An examination of several astrocytoma cell lines by immunoprecipitation for FAK followed by Western analysis using an anti-tyrosine monoclonal antibody revealed several cell lines in which FAK was phosphorylated while astrocytoma cells were grown on fibronectin as opposed to plastic (Fig. 6A). For the most part, the astrocytoma cell lines that showed activation of FAK through phosphorylations demonstrated an ability to bind to fibronectin in the cell attachment assays described above.

When GFAP-negative SF-126 astrocytoma cells were grown on plastic and then on fibronectin, there was minimal activation of phosphorylation of FAK. However, when the GFAP-positive SF-126 astrocytoma transfectants were grown on fibronectin, a marked upregulation of tyrosine phosphorylated FAK was demonstrated suggesting a preferential binding of the fibronectin receptor to fibronectin and activation of FAK accordingly (Fig. 6B).

Immunocytochemical Localization of FAK to Human Astrocytoma Cells

FAK was localized to sites of focal adhesions in human astrocytoma cells grown on fibronectin. Immunoreactivity was identified within the cells as fine punctate staining, or within the many long processes that developed from astrocytoma cells. In colocalization experiments with actin, these punctate structures were associated with actin stress fibers (Fig. 7A, B).

DISCUSSION

We have shown that astrocytoma cell lines express various integrin subunits by RT-PCR, and integrin heterodimers by immunoprecipitation analyses. The β1 integrin subunit was expressed by all astrocytoma cell lines by RT-PCR. Only 3 cell lines expressed the α2 integrin subunit, and the α3 subunit was expressed by all cell lines
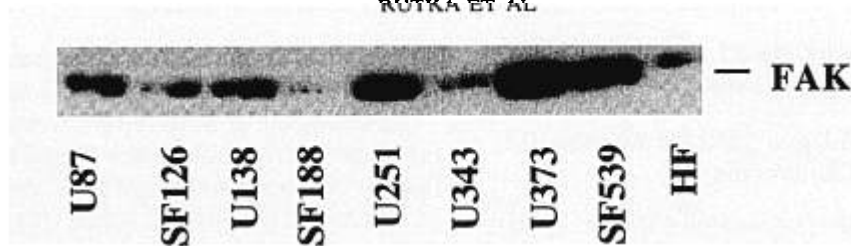
Fig. 3. Western blot analysis of FAK expression in astrocytoma cell lines. There is variable expression of the 125 kDa FAK protein on western analysis. SF-188 and U343 demonstrate the least amount of FAK protein, whereas U373 and U251 the most. HF = human fibroblasts.

except SF-188. The U87 MG astrocytoma cell line demonstrated the greatest repertoire of integrin subunit expression by RT-PCR. By immunoprecipitation analysis, the fibronectin receptor ($\alpha5\beta1$ integrin heterodimer) and the vitronectin receptor ($\alpha v\beta3$) were identified in several cell lines. For the most part, the results from the RT-PCR analysis correlated with those from immunoprecipitation in that the presence of integrin subunit expression by RT-PCR was predictive of those cell lines that would express detectable heterodimers. Furthermore, the integrin heterodimers we were able to demonstrate were functionally active when cell attachment to matrix studies were considered.

The ability of integrins to modify cell growth, differentiation, and adhesion appears to occur by 2 general pathways. "Outside-in" signaling involves the binding of an ECM-derived ligand to the integrin receptor (14, 15). This complex is formed from interlocking cytoskeletal components, and includes elements involved in intracellular signaling pathways. Outside-in signaling involves intracellular signals that alter integrin receptor conformation and affinity for ECM ligands (14). As one example, Zhang et al have shown that expression of the constitutively active GTP-binding protein, R-Ras, induces cells in suspension to adhere to ECM by converting integrins from a low-affinity to a high-affinity state (38). It is therefore not surprising that evidence is mounting implicating integrins in the pathogenesis of cancer, a disease in which alterations in cellular growth, differentiation, and adhesive characteristics are defining features. Based on these features, it has been postulated that changes in integrin expression would be found in a wide variety of human tumors. Indeed, immunohistochemical studies suggest that many tumors show loss or decreased expression of those integrins that may be essential for maintenance of stable adhesion and tissue organization (39–45). Conversely, integrins involved in migration through the ECM are increased (39, 46–48).

There have been several reports on the expression of integrins in human brain and astrocytomas (26, 40, 46, 48–55). Gladson and Cheresh showed that the $\alpha v\beta3$ integrin was the only vitronectin receptor identified in glioblastoma multiforme in situ (40). Paulus et al used immunohistochemical techniques to show that integrin

expression in normal astrocytes is dependent upon their immediate external environment (54). Parenchymal astrocytes, which were not in contact with mesenchymal cells, stained positively for only 2 integrin subunits, $\alpha3$ and $\beta1$. Perivascular astrocytes surrounding cerebral vascular structures and subpial astrocytes were positive for $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$ and $\beta4$ integrins (54). In anaplastic astrocytomas, $\alpha2$, $\alpha3$, $\alpha6$, and $\beta1$ integrins have been reported at levels comparable to those of normal astrocytes with the exception of $\alpha3$ and $\beta1$, which are increased (54). In glioblastoma multiforme, $\alpha2$, a3, $\alpha6$, αv, $\beta1$ and $\beta3$ were detected, with $\alpha3$, αv and $\beta1$ being expressed at increased levels compared to normal astrocytes (54). Interestingly, the microvasculature of glioblastoma multiforme has been shown to express the $\alpha v\beta3$ integrin (56).

Several astrocytoma cell lines have also been examined for integrin expression. The results from a number of laboratories have shown that astrocytoma cell lines commonly express $\alpha3$, αv, $\beta1$, $\beta3$ and $\beta4$ integrins (45, 46, 50, 51, 53, 54, 57). Giese et al recently showed that SF-767 astrocytoma cells use different integrins to adhere to and migrate along tenascin as a substrate (53). Our results are similar to those published in the literature. We observed $\beta1$ and $\alpha3$ integrin mRNA in virtually all astrocytoma cell lines. Levels of $\alpha2$, $\alpha5$, and $\beta3$ mRNA were present but low in most astrocytoma cell lines. However, unlike many of the previous studies in which RT-PCR was used to examine integrin subunit transcription levels or blocking antibodies to indirectly determine integrin subunits critical for cell attachment, we also employed immunoprecipitation techniques to study the heterodimer representation of integrins at the cell surface. From these studies, we were able to determine that the $\alpha5\beta1$ fibronectin receptor and the $\alpha v\beta3$ vitronectin receptor were present as heterodimers in many of our astrocytoma cell lines. Malek-Hedayat and Rome showed that the rat C6 glioma cell line expressed multiple integrin heterodimers by immunoprecipitation including $\alpha1\beta1$, $\alpha5\beta1$, $\alpha3\beta1$, and $\alpha v\beta5$ (58). However, to our knowledge, there have been few previous reports of integrin heterodimer expression in human astrocytoma cell lines. In one report, U251 MG astrocytoma cells were shown to express the $\alpha v\beta3$ integrin heterodimer by immunoprecipitation (40).
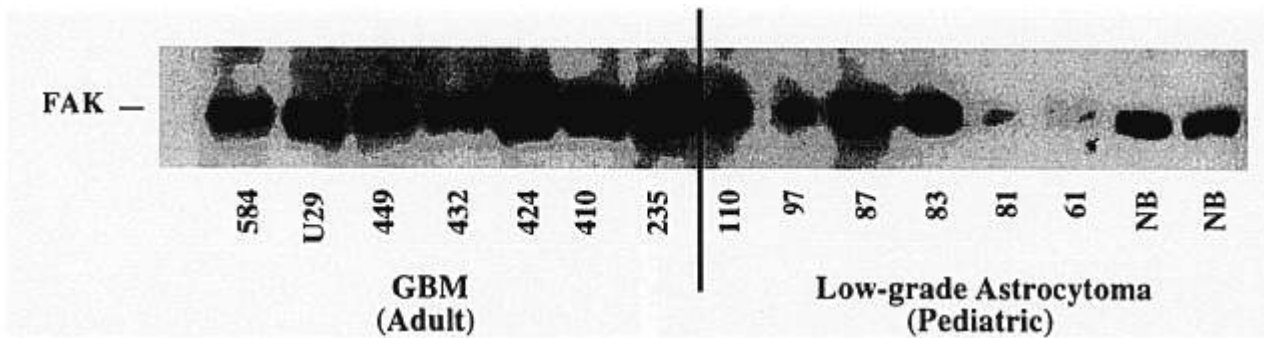
Fig. 4. FAK expression in human brain tumor samples. By western analysis, low-grade astrocytomas (juvenile pilocytic astrocytomas, grade I) demonstrated a more faint 125 kD FAK protein band than did the higher grade, glioblastoma multiforme (GBM, grade IV) specimens. Interestingly, some low-grade astrocytomas expressed FAK very minimally (e.g. tumor numbers 81 and 61). FAK protein is also expressed in specimens of nontumorous brain tissue (NB).

In another study, Haughland et al showed that the GaMG astrocytoma cell line expressed the α3β1 integrin heterodimer (50). Based on the information from our cell attachment assays, the integrin heterodimers we observed are likely functional as all cell lines examined demonstrated increased attachment to both human fibronectin and vitronectin as substrates compared to plastic alone. Furthermore, our studies with anti-integrin blocking antibodies showed that astrocytoma cells could be inhibited from binding to specific ECM components.

While the expression of FAK in the mouse central nervous system has been described (59), there have been no previously published reports on FAK expression in human astrocytomas. In the report by Grant et al, FAK was not restricted to focal adhesion contacts at astroglia, but was also found on axons and dendrites of neurons (59). Interestingly, in the same report, FAK was described as associating with GFAP in double immunohistochemical stains (59). Recently, Burgaya et al (60) showed that FAK mRNA derived from rat brain is alternatively spliced leading to the expression of several splice isoforms. As some of these isoforms have increased autophosphorylation activity, these authors have suggested that FAK may have specific properties in neurons. Using immunocytochemistry, we showed that FAK was present in human astrocytoma cells, and that FAK typically colocalized with actin stress fibers. Our study has shown that FAK is variably but quite ubiquitously expressed in human astrocytoma cell lines, and in several primary human astrocytoma specimens. In contrast to the report by Owens et al in which it was shown that overexpression of FAK correlated with tumor invasiveness (61), our study of FAK expression in low- and high-grade astrocytoma specimens revealed only a trend towards increased FAK in the high-grade astrocytic neoplasms.

We have demonstrated that FAK can be phosphorylated when astrocytoma cells bind to a fibronectin matrix, suggesting that an ECM:integrin-mediated signaling pathway is operational through FAK. Two lines of evidence from our study point toward the presence of a cytoskeletal-induced signaling pathway through integrins. In the first, antisense GFAP-transfected U251 human
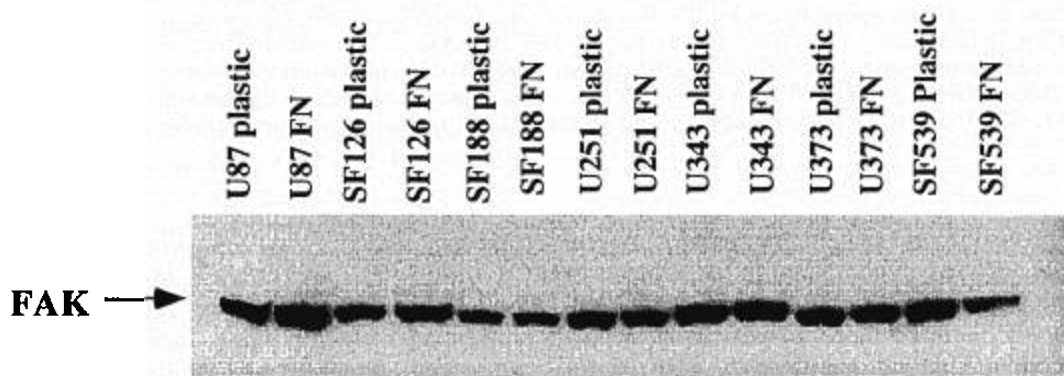
Fig. 5. FAK expression in astrocytoma cell lines grown on plastic (P) versus fibronectin (FN). There is variable expression of FAK among the different cell lines, but little if any difference in FAK expression when cells are grown on plastic (P) vs fibronectin (FN). The only cell line that showed an increase in FAK expression on FN versus P was U87 MG. SF-539 astrocytoma cells showed a decrease in FAK expression on FN when compared to plastic.
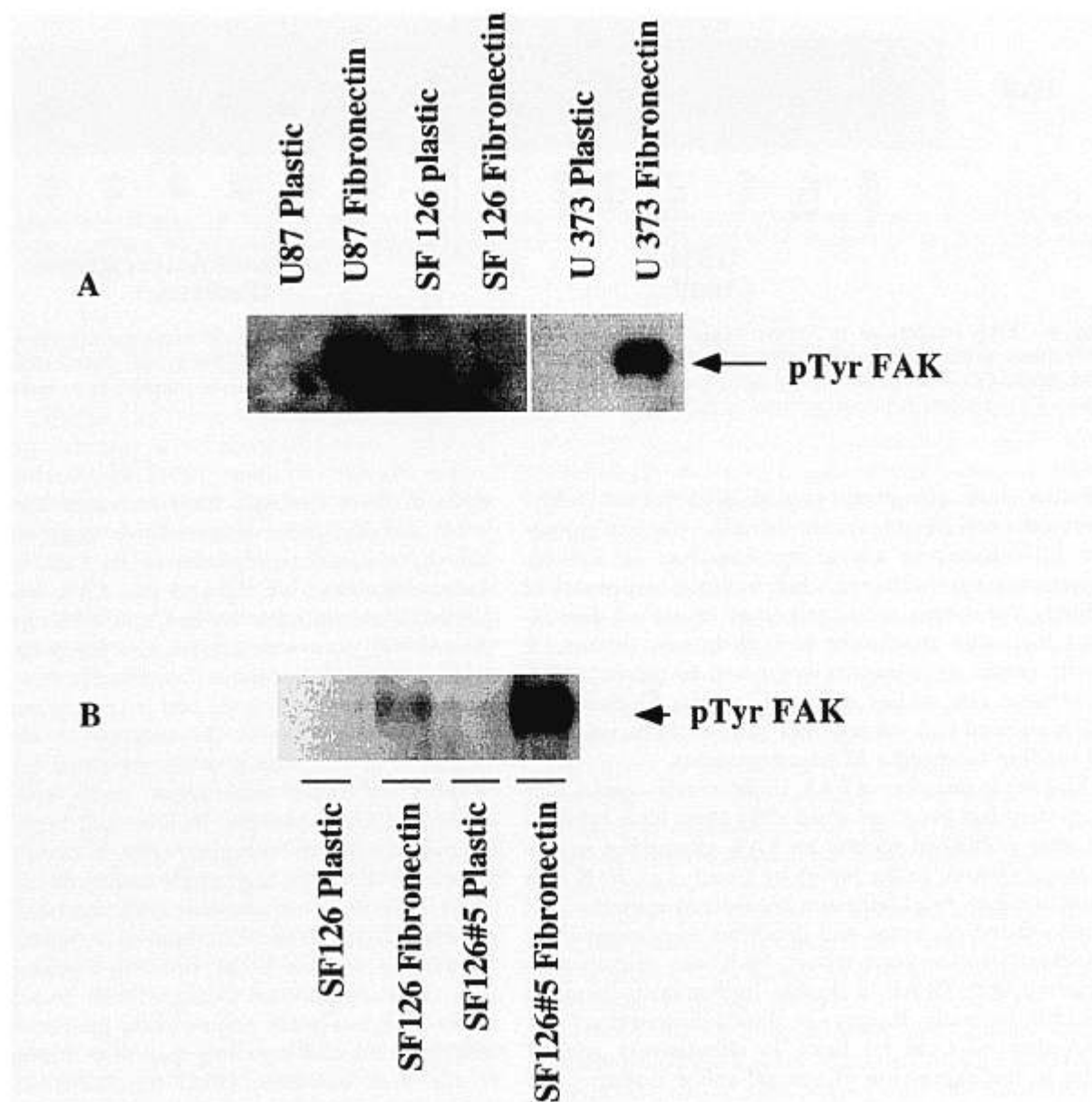
Fig. 6. Phosphorylation of FAK in human astrocytoma cell lines. A) U87 MG and U373 MG astrocytoma cell lines grown on fibronectin show tyrosine phosphorylation by IP-western analysis. Interestingly, SF-126 astrocytoma cells do not show phosphorylation of FAK on fibronectin. B) GFAP-transfected SF-126 astrocytoma cells demonstrate marked upregulation of FAK phosphorylation when grown on a fibronectin matrix compared to plastic and to nontransfected cells. (P = plastic; FN = fibronectin; SF126#5 = GFAP-transfected SF-126 astrocytoma cells, clone #5.) In this study, the antibodies used were polyclonal antibodies to FAK (C-20) and to tyrosine phosphorylated proteins (PY20) (Santa Cruz Biotechnology, Inc.).

astrocytoma cells were found to increase their β1 integrin expression by RT-PCR when compared to the controls or parental cell line. The antisense-GFAP transfectants have been previously shown to be more invasive than the parental cell line when Matrigel was used as a barrier for invasion (11). Interestingly, Paulus et al have shown that diffuse brain invasion of glioma cells requires β1 integrins (49). In their report, inducible expression of antisense β1 integrin was achieved using the tetracycline

repressor system in rat C6 glioma cells (49). Typically, C6 glioma cells are diffusely infiltrative of normal brain tissue in nude mice bearing intracranial tumors. When antisense β1 integrin was induced in C6 glioma cells, the tumor cells remained well delineated from normal brain and did not show invasive characteristics (49). These data support our findings in antisense-GFAP transfected U251 MG astrocytoma cells in which β1 integrins are upregulated, and which are more invasive than the parental cell
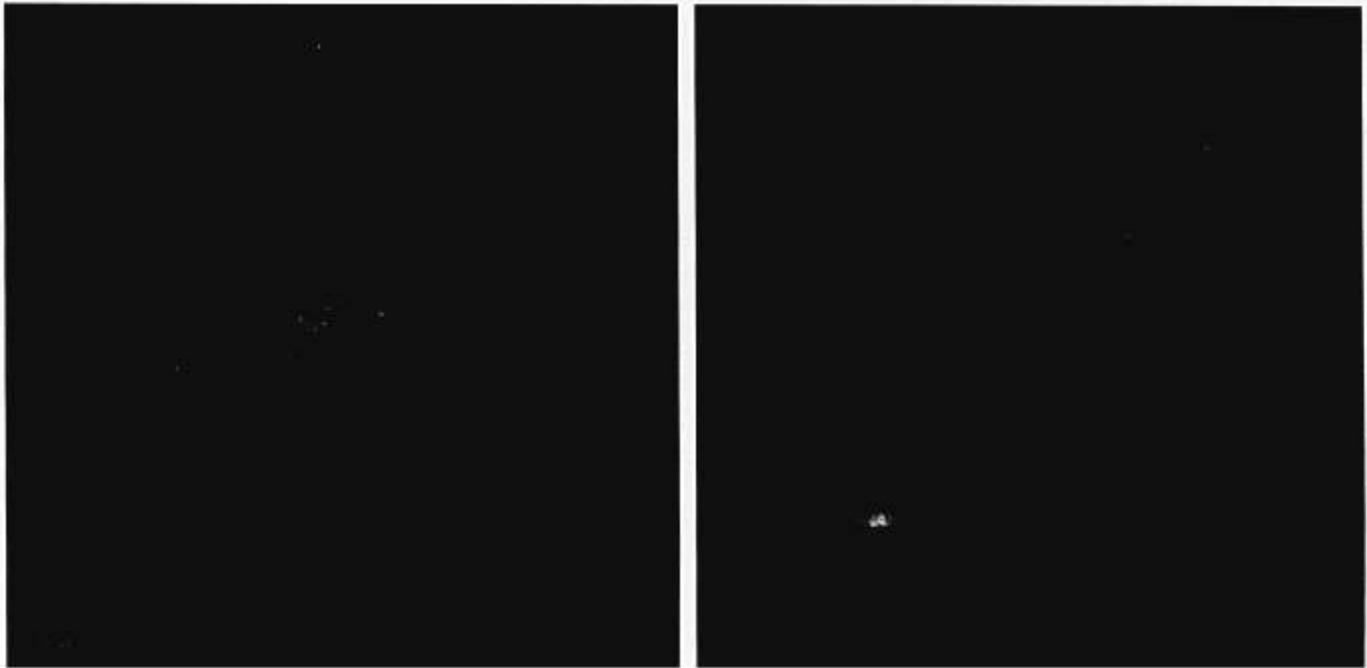
Fig. 7. Immunocytochemical localization of FAK and actin. A) Confocal image of FAK and actin in the U343 MG-A human astrocytoma cell line. FAK (green punctate structures) was found predominantly in association with actin stress fibers (red). B) FAK and actin colocalization in the U251 MG astrocytoma cell line. As with the U343 MG-A astrocytoma cell line, FAK (green punctate structures) was colocalized with actin stress fibers (red). Picture width in both A) and B) is 200 μm.

line. Why β1 and β3 integrins were upregulated in antisense-GFAP-transfected U251 MG astrocytoma cells and not αv integrin is an interesting but unresolved question at this time.

In the second, GFAP-transfected SF-126 human astrocytoma cells were shown to overexpress the phosphorylated form of FAK when these cells were placed on a fibronectin matrix. These results are of interest because they suggest that manipulations of the astrocytoma cytoskeleton can bring about signaling changes that channel through integrins and focal adhesion sites leading to activation of key kinases such as FAK. These data also support the existence of a separate "inside:out" signaling pathway in human astrocytoma cells. It is probable that GFAP-transfection in GFAP-negative astrocytoma cells leads primarily to a change in cell morphology brought about by cytoskeletal reorganization, cytoplasmic process and focal adhesion site formations. Precisely how the intermediate filament, GFAP, brings about such complex cellular rearrangements and signaling cascades is the focus of our current investigations.

ACKNOWLEDGMENTS

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