Focal Adhesion Kinase Is Expressed in the Angiogenic Blood Vessels of Malignant Astrocytic Tumors in Vivo and Promotes Capillary Tube Formation of Brain Microvascular Endothelial Cells

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ABSTRACT

Purpose: Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that has been shown to promote proliferation, migration, and invasion of several cell types in vitro, and we have shown recently that FAK promotes proliferation of malignant astrocytoma cells in vivo. To determine the role of FAK in angiogenesis in malignant astrocytic tumors, we investigated the expression and function of FAK in brain endothelial cells.

Experimental Design: We characterized the expression of FAK and activated FAK in endothelial cells by immunohistochemistry. We also determined the function of FAK in brain microvascular endothelial cells by transfecting these cells with a dominant interfering form of FAK (FRNK) or a mutant FRNK (Leu-1034 to Ser) and assessed the effect on capillary tube formation and cell migration.

Results: We found that FAK was expressed in the endothelial cells of grade III (4 of 9 samples) and IV (9 of 10 samples) astrocytoma biopsies but not in the endothelial cells of normal brain (0 of 9 samples) and not in grade I (0 of 5 samples) or II (0 of 4 samples) astrocytoma biopsies. Furthermore, we found that both FAK and activated FAK were expressed in the endothelial cells in malignant astrocytoma tumors propagated intracerebrally in the severe combined immunodeficient mouse brain. As expected, immunofluorescence analysis showed FRNK protein to localize to focal adhesions, whereas mutant FRNK protein did not. FRNK-transfected endothelial cells showed a 55% reduction in branched tube formation and a 40% reduction in tube length when propagated in three-dimensional collagen gels, compared with cells transfected with the mutant FRNK construct. Furthermore, FRNK-transfected cells showed a 35–50% reduction in haptotactic migration toward fibronectin and collagen, compared with mutant FRNK-transfected cells.

Conclusions: These data suggest that FAK promotes angiogenesis and that this occurs, at least in part, through the promotion of endothelial cell migration.

INTRODUCTION

Tumor angiogenesis, necessary for tumor cell invasion and proliferation, heralds an aggressive phase in the biology of a tumor and typically correlates with increased tumor growth and metastasis (1). In biopsy tissue from astrocytic neoplasms, proliferation of microvascular endothelial cells or angiogenesis has long been recognized as a histological indicator of high-grade malignancy (2). Angiogenesis can be broken down into several overlapping phases (3). The first phase (known as initiation) begins when vascular endothelial cells are activated by angiogenic cytokines. The second phase (known as proliferation/invasion) occurs when activated endothelial cells form a vascular sprout, requiring endothelial cell invasion and proliferation. The second phase also requires the release of proteolytic enzymes, which degrade the extracellular matrix, and the expression on the activated endothelial cells of integrin receptors, which interact with the extracellular matrix and thereby regulate and facilitate migration and invasion. In the third phase (known as maturation) the vascular sprout develops a lumen (tube formation).

A growing body of research has focused on the interactions between activated endothelial cells and the extracellular matrix, demonstrating an important role for integrin cell adhesion receptors in vascular sprouting and tube formation (1, 3). In high-grade astrocytic neoplasms, we and others (4, 5) have reported the up-regulation of integrins αvβ3, αvβ5, αvβ3, and lymphocyte function associated-1 antigen on the microvascular endothelial cells. Integrin receptors that connect the extracellular matrix with the inside of the cell serve as gateways to transmit biological signals from the extracellular matrix into the cell through the signals generated at adhesion structures (6). These signals regulate cell migration, proliferation, and gene transcription (6). One molecule involved in early integrin signaling is the cytoplasmic tyrosine kinase FAK, which has been

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4 The abbreviations used are: FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; HA, hemagglutinin; mAb, monoclonal antibody; SCID, severe combined immunodeficient.
shown to regulate cell migration, proliferation, survival, and invasion in some cell types (6–8). Integrin recognition of an extracellular matrix ligand typically results in the clustering of the integrin in the cell membrane and, in a temporally related manner, the autophosphorylation of FAK on tyrosine 397 (activation), followed by the formation of focal adhesions at the submembranous region of the cell (6, 7). Activation of FAK can also occur with growth factor and cytokine stimulation of cells; for example, other investigators have shown that stimulation with the angiogenic vascular endothelial cell growth factor or FGF-2 results in FAK activation (6, 7, 9, 10). FAK localization to focal adhesions is thought to be necessary for FAK signaling (7, 11), and overexpression of the noncatalytic COOH-terminal domain of FAK (known as FRNK) functions as a dominant interfering form of FAK by competing with FAK for localization to focal adhesions (12, 13).

The ability of FAK to promote cell migration was initially suggested when fibroblasts isolated from FAK knockout mice were demonstrated to exhibit reduced cell motility in culture (14) and reexpression of FAK in these null cells restored cell migration (15). Other investigators have shown that the signaling pathway by which FAK promotes cell migration in Chinese hamster ovary and COS cells requires the binding of the adapter protein p130CAS to FAK, Src phosphorylation of p130CAS, and Crk binding to p130CAS (16, 17). The dominant interfering form of FAK, FRNK, reduces cell migration of fibroblasts and prostate carcinoma cells and reduces epidermal growth factor-stimulated motility of lung adenocarcinoma cells in vitro (8, 18, 19). FAK also promotes MMP-2 and MMP-9 secretion, and FRNK transfection of A549 lung adenocarcinoma cells results in decreased levels of MMP-9 secreted into the media and in decreased invasion (8, 20).

The ability of FAK to promote cell migration, proliferation, and invasion suggested that FAK could be necessary for endothelial cell sprouting and tube formation. To our knowledge, no study investigating the effect of inhibiting FAK function on microvascular endothelial cell tube formation has been reported previously. In this study, we tested the hypothesis that FAK is expressed by angiogenic endothelial cells in malignant astrocytic tumors and that it is necessary for brain microvascular endothelial cell tube formation. We show that microvascular endothelial cells in malignant astrocytoma tumor biopsy samples express elevated levels of FAK and that a dominant interfering form of FAK (FRNK) inhibits endothelial cell migration and tube formation of brain microvascular endothelial cells.

**MATERIALS AND METHODS**

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded blocks of surgically resected brain tissue were obtained from the University of Alabama at Birmingham Hospital, in accordance with the University Human Tissue Committee policies, Institutional Review Board exemption X980409003. The tumors were independently graded according to the WHO classification of brain tumors (2). Grade IV astrocytic neoplasms are characterized histologically by endothelial cell proliferation (angiogenesis), which may also be seen in the grade III astrocytic tumors (2). The nonneoplastic brain biopsy samples were diagnosed as hippocampal sclerosis (seizure disorder), radiation gliosis, and normal brain. Immunohistochemistry was performed as described previously (5, 21), using rabbit anti-FAK IgG (Upstate Biotechnology, Inc., Lake Placid, NY), with normal rabbit serum IgG as a negative control, at a concentration of 10 μg/ml. Briefly, 5-μm sections were deparaffinized in xylene, rehydrated, subjected to antigen retrieval, digested with pepsin, and blocked in a 1% solution of hydrogen peroxide in methanol. Tissue sections were then subjected to nonspecific protein blocking in 1% BSA in PBS with 0.01% Tween 20 (22°C, 60 min), incubated with the primary antibody in 1% BSA in PBS with 0.01% Tween 20 (4°C, 12 h), washed, and reacted with a secondary antibody (22°C, 20 min), and this was followed by the biotin-streptavidin label and then 3,3′-diaminobenzidine substrate (kit from Bio-Genex, San Ramon, CA).

Immunohistochemistry on frozen sections from paraformaldehyde-fixed and frozen SCID mouse brain xenografts was performed as described above, with the following changes: all incubations were performed at 4°C; and tissue sections were briefly washed in PBS, permeabilized in 0.6% Triton X-100 in PBS for 10 min, blocked in 1% hydrogen peroxide in methanol, blocked for 1 h in 5% BSA in PBS with 0.01% Tween 20, and then incubated for 48 h with 5 μg/ml primary antibody. The slides were then processed as described above. The antibodies used were rabbit anti-FAK IgG, rabbit anti-FAK (pY397) phosphospecific IgG (BioSource International, Camarillo, CA), rabbit antimouse CD31/PECAM IgG (PharMingen, San Diego, CA) as a positive control, and mouse IgG as a negative control. The phosphorylated peptide antigen recognized by rabbit anti-FAK (pY397) phosphospecific IgG was purchased (BioSource International).

**U-251MG Malignant Astrocytoma SCID Mouse Xenografts.** CB17 SCID mice were obtained from the Fredericks Cancer Institute (Bethesda, MD). U-251MG cells, obtained from the American Type Culture Collection (Manassas, VA), were propagated as described previously (22), harvested with buffered EDTA, and washed, and 0.5 × 10⁶ cells (in 10 μl of PBS) were injected intracerebrally with stereotactic assistance into the right basal ganglia (22). At 14 days postinjection, the mice were euthanized, and the brains were harvested and fixed in 4% buffered paraformaldehyde (for 4 h), immersed in 6% sucrose solution (for 3 h), and then frozen and maintained at −70°C (22). The brains were serially sectioned and stored at −20°C until use.

**Electroporation of Endothelial Cells.** IBE mouse brain microvessel endothelial cells, a kind gift from Dr. Lena Claeson-Welsh (Ludwig Research Institute, Uppsala, Sweden), were propagated as described previously (23) in Ham’s F-12 media with 10% FCS, 20 units/ml murine IFN-γ, 50 μg/ml gentamicin, and 2.5 μg/ml amphotericin. These cells were isolated previously from the brain of the temperature-sensitive large T antigen transgenic mouse (23). HA-tagged FRNK and mutant FRNK (Leu-1034 to Ser) constructs were a kind gift from Dr. David Schlaper (Scripps Research Institute, La Jolla, CA; Ref. 18). IBE endothelial cells were harvested with trypsin and washed two times in 1× Ham’s F-12 media with 10% FCS, and then 20 μg of FRNK or the mutant FRNK construct were added to 1 × 10⁶ cells in 400 μl of complete media without antibiotics and electroporated at 960 μF, 170 mV, in a Bio-Rad Gene Pulser. After sitting at room temperature for 10 min, the cells were...
transferred to complete media and allowed to recover for 20 h (33°C, 5% CO₂).

Immunofluorescence Analysis. IBE cells were harvested with buffered EDTA at 20 h posttransfection and replated in complete media onto collagen-coated glass coverslips (33°C, 5% CO₂, 3 h). Subsequently, the cells were fixed, permeabilized, and double-labeled by sequential staining with primary and secondary antibodies as described previously (21). The antibodies used were mAb anti-HA IgG (20 μg/ml; Upstate Biotechnology), rabbit anti–β1 IgG directed toward the cytoplasmic tail (20 μg/ml; Chemicon International, Temecula, CA), antimouse Alexa-488 IgG (Molecular Probes, Temecula, CA), and antirabbit Alexa-594 IgG (Molecular Probes). Fluorescence was analyzed using a Nikon confocal microscope.

Capillary Tube Formation Assay. Collagen gels were prepared as follows (23): a solution of 1 part serum-free 10× Ham’s F-12 media, 8 parts type I collagen, and 2 parts 0.2 M NaOH was prepared on ice (pH 7.6). The collagen (200 μl) was aliquoted into a 48-well plate and allowed to gel for 1 h at 37°C. IBE brain microvascular endothelial cells were harvested, washed, counted, resuspended in serum-free 1× Ham’s F-12 media with 0.25% BSA, and then aliquoted (100,000 cells/cm²) along with 5 ng/ml FGF-2 onto the collagen gel and incubated at 33°C for 2 h. The media were aspirated, and a second collagen gel was poured over the cells. Finally, serum-free 1× Ham’s F-12 with 0.25% BSA was added to the top of the gel, and the three-dimensional collagen gels were incubated for 2 days (33°C, 5% CO₂). Branched tube formation was imaged with a Nikon video microscope using the ×20 objective and analyzed using the Universal Metamorph software (Universal Imaging Corporation, West Chester, PA). To determine the percentage of dead cells at 48 h, the gels were stained with 0.08% trypan blue (1.5 min), the stain was removed, the gels were photographed (ten ×20 fields), and the percentage of blue-stained cells was counted by a blinded investigator.

Western Blot Analysis. Cells were harvested and lysed in radioimmunoprecipitation assay lysis buffer with protease inhibitors (4°C, 10 min), centrifuged (35,000 rpm, 4°C, 1 h), and Western blotted as described previously (22), and the supernatant was stored at −70°C. Equivalent microgram of protein from each lysate was electrophoresed on a 7.5% disulfide-reduced SDS-PAGE gel, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), Western blotted with 1 μg/ml mAb anti-HA IgG, and stripped and reprobed with a 1:1000 dilution of mAb antiaxin (Sigma Chemical Co.). For semiquantitative analysis of band intensity, specific bands on the autoradiographs were subjected to densitometric analysis, and the background densitometric reading on the autoradiograph was subtracted.

Attachment and Migration Assays. IBE cells were harvested 20 h posttransfection, washed, and resuspended in serum-free Ham’s F-12 media with 1% BSA. For the attachment assays, 20,000 cells were plated onto collagen-, fibronectin-, or ovalbumin-coated wells, allowed to attach (33°C, 5% CO₂, 30 min), and washed twice with PBS; the attached cells were then fixed, stained, dried, and solubilized in acetic acid, and the absorbance for each well was determined in an ELISA reader at 600 nm, as described previously (24). Attachment to ovalbumin was subtracted. Conditions were assayed in replicates of four, and the data were analyzed and presented as the mean ± SE. For the migration assays, 20,000 cells in serum-free Ham’s F-12 media with 1% BSA were plated onto 8 μm filters coated on the bottom surface with collagen type I or fibronectin and allowed to migrate (33°C, 5% CO₂, 3 h) as described previously (22). Cells on the upper filter surface were removed, and the cells on the lower filter surface were fixed, stained, and counted. Conditions were assayed in replicas of four, and the data were analyzed and presented as the mean ± SE.

RESULTS

Expression of FAK in the Angiogenic Endothelial Cells of Malignant Astrocytoma Biopsy Samples. To determine the biological role of FAK in the angiogenic process, FAK expression in the endothelial cells of formalin-fixed and paraffin-embedded human astrocytoma tumor biopsies was determined by immunohistochemical analysis with rabbit anti-FAK IgG. Microvascular endothelial cell expression of FAK was restricted to grades III and IV astrocytic tumor biopsy samples (Fig. 1A; Table 1). In contrast, endothelial cells in nonneoplastic brain biopsy samples (Fig. 1C) and in grade I and II astrocytic tumor biopsy samples failed to express detectable levels of FAK protein by this technique. Also, as shown in Table 1, detectable FAK expression in astrocytic tumor cells was increasingly common with increasing tumor grade, consistent with our previous reports (22, 25).

Expression of FAK and Activated FAK in the U-251MG Human Malignant Astrocytoma Xenograft Propagated Intracerebrally in the SCID Mouse. To determine whether FAK was activated in the angiogenic endothelial cells of human malignant astrocytoma tumors, we used the intracerebral SCID mouse xenograft model, which we established previously (22). It was necessary to use an animal model to test for activated FAK because the brain tumors were optimally fixed for staining with the phosphospecific anti-FAK (pY397) antibody. FAK protein and activated FAK protein were expressed in the microvascular endothelial cells of these tumors (Fig. 2, C–F). Furthermore, anti-FAK (pY397) IgG staining was competed out by preincubating the antibody with the phosphorylated peptide antigen (Fig. 2, G and H), indicating specific staining of activated FAK in the endothelial cells in these tumors (Fig. 2, E and F). Endothelial cell positivity for FAK and activated FAK (pY397) by this technique was limited to 25–50% of microvessels in these tumors, indicating some variability in FAK expression and activation in tumor endothelial cells. Weaker tumor cell staining for FAK and activated FAK was also seen (Fig. 2, C–F).

Expression of FRNK in Brain Microvascular Endothelial Cells. To determine the role of FAK in angiogenesis, the dominant interfering form of FAK, FRNK, or mutant FRNK (Leu-1034 to Ser), which does not localize to focal adhesions, was transiently transfected into IBE mouse brain microvascular endothelial cells, and this was followed by recovery in complete media for 20 h. Western blot analysis with mAb anti-HA IgG (antibody directed toward the constructs tag) confirmed that FRNK and the mutant FRNK were expressed in the transfected
cells (Fig. 3A). FRNK and mutant FRNK were detected as bands of 48 kDa relative molecular mass (Fig. 3A, Lanes 1 and 2, respectively). To determine whether FRNK localized to focal adhesions and thus could inhibit FAK signaling, immunofluorescence analysis of transfected IBE cells was performed. Using mAb anti-HA IgG, we found that the HA-tagged FRNK readily localized to focal adhesions in IBE cells adherent to collagen for 3 h (Fig. 4B), whereas the mutant FRNK construct was found predominately in a diffuse pattern in the cytoplasm (Fig. 4D). The β1 subunit colocalized with FRNK to focal adhesions (Fig. 4, A and B, respectively) but not with the mutant FRNK (Fig. 4, C and D). mAb anti-HA IgG staining demonstrated that ≥90% of the transfected cells expressed the FRNK and the mutant FRNK proteins at 20 h posttransfection (data not shown).

Expression of FRNK in IBE Brain Microvascular Endothelial Cells Inhibits Capillary Tube Formation. To determine the role of FAK in capillary tube formation, FRNK-transfected IBE cells were examined for branched tube formation and tube length in three-dimensional collagen gels under serum-free conditions. In IBE cells expressing FRNK, we found a significant inhibition of branched tube formation (50%) and tube length (40%) at 2 days (Fig. 5, B, D, and E). In contrast, in IBE cells expressing mutant FRNK, no significant change in branched tube formation or tube length was found (Fig. 5, C–E). No increase in endothelial cell death was detected in the IBE cells expressing FRNK, compared with mutant FRNK, when propagated in the three-dimensional collagen gels [percentage of dead cells (X ± SE): FRNK-transfected cells, 2160 FAK Promotes Angiogenesis

Table 1  Expression of FAK in endothelial cells in various grades of astrocytic tumors and in nonneoplastic brain

Immunohistochemistry was performed as described in “Materials and Methods.” Nonneoplastic brain biopsy samples included mesial sclerosis, radiation gliosis, and normal brain. Astrocytic tumor biopsy samples were diagnosed according to the World Health Organization Classification (2). The staining intensity was graded as negative (−), positive or light brown (+), and strongly positive or dark brown (++). Negative (−) denotes no staining over the rabbit IgG used as a negative control. The criteria for positive staining was that ≥8% of the appropriate cell type or of blood vessels were stained. The numbers in parentheses indicate the percentage of astrocytic cells or of blood vessels staining with rabbit anti-FAK IgG.

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<td>Nonneoplastic brain</td>
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<td>Anaplastic astrocytomas</td>
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<td>Glioblastoma multiforme</td>
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Fig. 1  Expression of FAK in the microvessel endothelial cells of malignant astrocytic tumors. Immunohistochemistry was performed as described in “Materials and Methods.” A and B, grade IV astrocytic tumor (glioblastoma multiforme); C and D, nonneoplastic brain. A and C, rabbit anti-FAK IgG; B and D, normal rabbit serum IgG. Arrows denote endothelial cells. Magnification, ×20.
Fig. 2 Expression of FAK and activated FAK in the microvessel endothelial cells of malignant astrocytic tumors propagated intracerebrally in the CB17 SCID mouse. Immunohistochemistry was performed as described in “Materials and Methods.” A and B, antimouse CD31 IgG; C and D, rabbit anti-FAK IgG; E and F, rabbit anti-FAK (pY397) IgG; G and H, rabbit anti-FAK (pY397) IgG preincubated with the phosphorylated peptide antigen used to create this antibody. Arrows denote endothelial cells. Magnification: A, C, E, and G, ×10; B, D, F, and H, ×20.
This suggests FRNK had no detectable effect on cell survival under these conditions. These experiments indicate that the expression of FRNK inhibits capillary tube formation of brain microvascular endothelial cells.

Expression of FRNK in IBE Brain Microvascular Endothelial Cells Inhibits Haptotactic Cell Migration. To determine whether FAK was necessary for migration of the IBE brain microvascular endothelial cells, FRNK or mutant FRNK was transfected into IBE cells, and this was followed by recovery in complete media for 20 h and the haptotactic migration assay as described in "Materials and Methods." We found that the expression of FRNK protein significantly inhibited (35–50%) haptotactic migration toward collagen and fibronectin, whereas expression of the mutant FRNK protein did not alter cell migration (Fig. 3C). This inhibition of cell migration in the FRNK-transfected cells was not because of an inability of these cells to attach to collagen or fibronectin because expression of the FRNK or the mutant FRNK protein did not significantly alter IBE cell attachment to collagen or fibronectin at 1 h (Fig. 3D).

DISCUSSION

In this article we provide evidence suggesting that FAK promotes angiogenesis in malignant astrocytic tumors. We found an elevated expression of FAK in the microvascular endothelial cells in grade III and IV astrocytic tumors but not in the microvascular endothelial cells of grade I or II tumors and not in normal brain cells. Furthermore, when these studies were repeated on optimally fixed frozen brain tumors from an intracerebral SCID mouse xenograft model of malignant astrocytoma, FAK and activated FAK were detected at increased levels in the microvascular endothelial cells in the tumors, compared with the normal brain cells. To determine the role of FAK in capillary tube formation, a dominant interfering form of FAK, FRNK, and a mutant FRNK that does not localize to focal adhesions were transfected into IBE brain microvessel endothelial cells. FRNK-transfected endothelial cells showed a significant reduction in haptotactic migration (35–50%) and in branched tube formation and tube length (40–50%) when propagated in three-dimensional collagen gels.

Elevated expression of FAK and activated FAK in the microvascular endothelial cells of a tumor compared with the corresponding normal tissue has not been demonstrated previously, to our knowledge. Other investigators studying FAK expression in oral squamous cell cancer noted that FAK was expressed in the endothelial cells in these tumors (26). Because FAK has been shown to promote migration, invasion, and proliferation of some cell types in vitro and because these processes are necessary for angiogenesis, the elevated level of
FAK and activated FAK protein that we detect in angiogenic endothelial cells in malignant astrocytic tumors is consistent with these functions. FAK and activated FAK could not be detected in 100% of the microvascular endothelial cells in the xenograft tumors, suggesting some differential activation of the endothelium, probably because of regional differences in the proangiogenic factors found in the tumor microenvironment.

To determine the function of FAK in activated brain microvascular endothelial cells, we transfected a dominant interfering FAK construct (FRNK) into these cells and assessed capillary tube formation. A three-dimensional collagen gel matrix was chosen for the in vitro capillary tube assays because other investigators have shown that the collagen receptor integrin αβ1 is up-regulated on brain microvascular endothelial cells in malignant astrocytomas (4). We found that the expression of FRNK, but not of the mutant FRNK protein, significantly blocked branched tube formation and tube length in an in vitro serum-free three-dimensional model of angiogenesis. These data are supported by the observation that the tubulogenic activity associated with a constitutively active form of the Flt-1 kinase (a vascular endothelial growth factor receptor) in embryonic fibroblasts is dependent on FAK (27). Because endothelial cell migration is necessary for tube formation, we investigated the effect of FRNK transfection on migration and found that the expression of FRNK protein (but not of the mutant FRNK protein) significantly inhibited haptotactic migration of IBE brain microvascular endothelial cells toward collagen and fibronectin in a 3-h assay. There is substantial evidence that FAK is necessary for directed cell migration in vitro of other cell types (7, 16, 17, 18, 22), providing support for our finding regarding brain microvascular endothelial cells.

The mechanism responsible for FAK activation during the process of endothelial cell sprouting and tube formation is likely multifactorial. Integrin receptor engagement likely contributes to FAK activation in our capillary tube formation assay because integrin receptor engagement typically leads to FAK activation (6, 7). In the serum-free capillary tube formation assay used in this study, FGF-2 was the proangiogenic stimulus, and no other exogenous growth factor or cytokine was added. FGF-2 has been reported to activate FAK in smooth muscle cells (10) and to promote chemotactic migration of endothelial cells (28). Therefore, FGF-2 is likely contributing to FAK activity and the microvascular endothelial cell migration and sprouting in our three-dimensional model. In addition, autocrine events occurring in the three-dimensional collagen gels may promote FAK activation, cell migration, and sprouting.

Other mechanisms by which FAK could potentially promote capillary tube formation include promotion of MMP-2 or MMP-9 secretion or promotion of endothelial cell survival because FAK promotes MMP-2 and MMP-9 secretion and cell survival in some cell types propagated as a monolayer in culture (6, 8, 20). We found no increase in cell death in the IBE cells transfected with FRNK when propagated in the three-dimen-

Fig. 4 FRNK localizes to focal adhesions in IBE brain microvascular endothelial cells. IBE brain microvascular endothelial cells were transfected with either FRNK or mutant FRNK or were mock transfected. The cells were allowed to recover for 20 h and then harvested and replated onto collagen-coated coverslips, and this was followed by double-label immunofluorescence analysis, as described in “Materials and Methods.” A and B, FRNK-transfected rabbit anti-β1 IgG and mAb anti-HA IgG, respectively. C and D, mutant FRNK-transfected rabbit anti-β1 and mAb anti-HA IgG, respectively. Arrows denote focal adhesions.
sional collagen gels, and in future studies we will investigate the potential role of FAK in promoting MMP-2 or MMP-9 secretion by the brain microvascular endothelial cells.

In summary, our data suggest that FAK promotes angiogenesis in malignant astrocytic tumors and that the mechanism by which FAK promotes angiogenesis is a result of, at least in part, its promotion of endothelial cell migration. FAK is potentially a useful therapeutic target in the angiostatic treatment of malignant astrocytic tumors in patients.

REFERENCES


Fig. 5 Expression of FRNK inhibits capillary tube formation of IBE brain microvascular endothelial cells. IBE brain microvascular endothelial cells were transfected with FRNK (A), transfected with mutant FRNK (B), or mock transfected (C), allowed to recover, and plated 20 h later onto a collagen gel with 5 ng/ml FGF-2. Two h later, the media were removed, and a second collagen gel was poured over the cells, and this was followed by serum-free media and incubation for 2 days (33°C, 5% CO₂). D, histogram of the branched tube formation; E, histogram of the tube length. D and E were both analyzed using the Universal Metamorph software. Conditions were assayed in replicas of four, and the branched tube formation and tube length were analyzed in five fields at ×10 magnification in each replica. Data were analyzed and presented as the mean ± SE.
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