ABSTRACT

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that on activation generates signals that can modulate crucial cell functions, including cell proliferation, migration, and survival. In vitro, overexpression of FAK has been shown to promote cell proliferation by signaling through the Ras/mitogen-activated protein kinase cascade in several cell types. We have shown previously that overexpression of exogenous FAK lacking alternative splicing in malignant astrocytoma clones injected intracerebrally into SCID mouse brains promotes tumor cell proliferation. Here, we show that in anaplastic astrocytoma biopsy samples, FAK is expressed as an unspliced variant and migrates with a faster mobility similar to that observed in embryonic brain. Compared with nonneoplastic adult brain biopsies, the levels of FAK protein are elevated as are its levels of activation as assessed by autophosphorylation and overall tyrosine phosphorylation. The activity of Src kinase in these tumors is also elevated, as well as the activity of Src kinase associated with FAK; the latter may result in enhanced Src kinase phosphorylation of FAK. Phosphorylated Shc is associated with FAK in the anaplastic astrocytoma biopsy samples and in astrocytoma cells overexpressing FAK in vitro but not in nonneoplastic brain biopsy samples. Elevated extracellular signal-regulated kinase-2 activation and elevated expression of cyclins D and E are also found in anaplastic astrocytoma biopsy samples. These data provide evidence that the increased FAK activity in these tumors contributes to phosphorylation of Shc and likely to the promotion of Ras activity, extracellular signal-regulated kinase-2 activation, and cell proliferation in vivo.

INTRODUCTION

Malignant astrocytomas, Grades III and IV, are highly proliferative and invasive brain tumors with a dismal prognosis (3- and 1-year median survival, respectively; Ref. 1). A knowledge of the mechanisms that lead to cell proliferation and migration in these high-grade tumors would provide a framework for the design of more effective therapeutic strategies. One molecule that appears to be involved in early cell surface receptor signaling of proliferation, migration, and survival is the cytoplasmic tyrosine kinase, FAK (2). FAK is activated upon integrin receptor engagement with the extracellular matrix or by ligation of several other types of cell surface receptors (2). In nonneoplastic cells, integrin clustering in the membrane leads to autophosphorylation of FAK at tyrosine 397, which provides a docking site for the tyrosine kinase, Src, through Src’s SH2 domain (3, 4). Src subsequently phosphorylates FAK at several sites, including tyrosine 925; in fibroblasts, phosphorylation of tyrosine 925 can result in the recruitment of the Grb2 adapter protein to FAK, leading to the activation of Ras and mitogen-activated protein kinase (5–7). Several other molecules can bind to tyrosine 397 of FAK after its autophosphorylation, such as phosphatidylinositol 3-OH kinase and Shc, which can lead to the activation of Akt and Ras, respectively (7–9).

Evidence that FAK plays a role in cell proliferation and survival has come for the most part from studies of cell lines propagated in vitro. Overexpression of FAK in Chinese hamster ovary cells resulted in an accelerated G1 to S cell cycle transition and increased cyclin D expression, suggesting a role for FAK in the promotion of cell proliferation (10). Several other lines of evidence from studies of nongliarial cell lines propagated in vitro suggest that FAK promotes cell survival, e.g., antibody or peptide blocking of FAK binding to integrins resulted in rapid cell death of fibroblasts (11), fibronectin survival signals transduced by FAK suppress p53-regulated apoptosis in fibroblasts and endothelial cells (12), and a constitutively active form of FAK rescued epithelial cells from programmed cell death (13). FAK has also been implicated in the promotion of cell migration in cell lines propagated in vitro (14, 15). Cell motility studies of fibroblasts from FAK-knockout mice indicate that such cells exhibit reduced cell motility in culture (16), and re-expression of FAK in FAK-null fibroblasts restored cell migration (17). Studies of rat tissue have shown that the FAK message is expressed in all organs; however, in normal brain and testes, it is spliced alternatively and encodes three additional short exons around the autophosphorylation site (tyrosine 397), which have been termed Boxes 28, 6, and 7 (18). In addition, FAK mRNA from adult rat brain containing these splice variants migrates with a slower mobility on SDS-PAGE (~129 kDa; Ref. 18). In contrast, FAK protein in the embryonic rat brain migrates with a faster mobility (~125 kDa; Ref. 19). Studies investigating the biological role of FAK in tumors in vivo have for the most part been limited to immunohistochemical studies of tumor biopsy samples. Such studies have demonstrated elevated expression of FAK in colon, breast, gastrointestinal, and ovarian carcinoma (20–23). Our early studies had established that expression of integrins αvβ3 and αvβ5 is up-regulated on malignant astrocytes in both patient biopsies and in an intracerebral xenograft model of human malignant astrocytoma (24, 25). More recently, we have shown that attachment of malignant astrocytoma cells to several ligands in vitro results in the phosphorylation of FAK (9) and that overexpression of FAK in U-251 MG human malignant astrocytoma cells resulted in a 2-fold increase in soft agar growth and increased migration in vitro (26). On propagation of tumors in the SCID mouse brain after stereotactic injection, these clones that overexpressed FAK exhibited a 1.8–2.8-fold increase in tumor cell proliferation in vivo that was independent of apoptosis (26).

Interestingly, malignant astrocytomas (both grades III and IV) demonstrate a high level of Ras-GTP activity in vivo; however, oncogenic mutations affecting Ras activity are not found (27). Therefore, unlike some other human cancers that are marked by Ras gain-of-function mutations, the increased Ras activity in malignant astrocytomas must occur secondary to upstream signals. In vitro studies of malignant astrocytoma cells have implicated Shc phosphorylation and the subsequent recruitment of the Grb2/SOS complex in the activation of Ras-GTP (28). As FAK is a potential upstream activator of Ras, we investigated the expression, activity, and signaling of the FAK molecule in malignant astrocytic tumor biopsy samples. Here we report that anaplastic astrocytoma tumor biopsy samples...
FAK SIGNALING IN TUMORS IN VIVO

(grade III) show an increase in FAK autophosphorylation and overall tyrosine phosphorylation, as well as increased Src kinase activity, as compared with nonneoplastic brain samples. In addition, we demonstrate the coimmunoprecipitation of endogenous FAK protein with endogenous phosphorylated Shc in these tumor biopsy samples but not in nonneoplastic brain samples, which correlates with elevated ERK-2 activation in these tumors. These data provide the first in vivo evidence that endogenous FAK activity and signaling, through its coupling with Shc, could contribute to the phenotype of malignant astrocytomas by promoting Ras activity and subsequently ERK-2 activity.

MATERIALS AND METHODS

Antibodies and Reagents. Nonneoplastic brain, grade I pilocytic astrocytoma tumors, and grade III anaplastic astrocytoma tumors were obtained from the Brain Tumor Bank at The University of Alabama at Birmingham Hospital in accordance with the University Human Tissue Committee policies. Institutional Review Board exemption X980409003. Tumors were diagnosed and classified according to the criteria of the revised WHO Classification of Brain Tumors (1). Nonneoplastic brain and tumor samples were frozen and stored at −70°C and thawed immediately before detergent lysis and analysis. U-251 MG and U-87 MG human malignant astrocytoma cells were obtained from the American Type Culture Collection, maintained, and propagated in complete medium with 10% fetal bovine serum, as described (25). Primary human astrocytes were isolated as described (29).

The following antibodies were purchased as indicated: rabbit anti-FAK IgG, mAb anticyclin D1, rabbit anti-ScrpY146 IgG, and rabbit antiphosphotyrosine IgG from Upstate Biotechnology (Lake Placid, NY); rabbit anti-FAKIpY397) IgG from BioSource International (Camarillo, CA); mAb anti-actin ascites from Sigma Chemical Co. (St. Louis, MO); mAb anti-PY20 from ICN Biomedical (Irvine, CA); rabbit antiphospho-ERK IgG from Cell Signaling-New England Biolabs (Beverly, MA); and mAb anti-c-Src, mAb anti-Shc, mAb antiphospho-JNK, mAb anti-JNK, mAb anti-FAK COOH terminus, rabbit anti-FAK NH2 terminus IgG, and rabbit anti-ERK IgG from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). mAb anticyclin E was a kind gift from Dr. Tom Broker (University of Alabama at Birmingham, Birmingham, AL).

Western Blotting and Immunoprecipitations. Human brain and tumor samples were thawed on ice and lysed using a Polytron in 1% NP-40 lysis buffer containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, and 20 mM Tris-HCl (pH 8.0), with the following protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM sodium vanadate) and centrifuged (35,000 rpm, 1 h, 4°C), and the supernatant was stored at −70°C. For Western blots, equivalent μg of protein from each lysate were electrophoresed on a 6.5% or 12% SDS-PAGE gel, transferred to Immobilon-P membrane (Millipore Corp.), blocked with 5% BSA (3 g, 22°C), and reacted with primary antibody overnight at 4°C, followed by reaction with 0.025 g/ml horseradish peroxidase-conjugated goat antirabbit or goat antirabbit IgG (Bio-Rad) for 45 min at 22°C, and the membrane was developed with the Amersham enhanced chemiluminescence chemiluminescent system (Pharmacia Biotech), as described previously (26). For quantitative analysis of band intensity, specific bands on the autoradiograms were subjected to densitometric analysis, and the background densitometric reading on the autoradiograph subtracted. For commonoprecipitation analysis, equivalent μg of protein from each sample were incubated with Sepharose-coupled antibody overnight at 4°C, washed, subjected to 6.5% or 12% SDS-PAGE, transferred to Immobilon, and Western blotted.

Nonquantitative RT-PCR. Human brain and tumor samples were thawed, and total RNA was extracted according to Qiagen RNaseasy (Valencia, CA). Equivalent ng of total RNA from each sample were subjected to reverse transcription, followed by 30 cycles of PCR using the following primers: NH2 terminus: 5' TTAGCAGTACTAACCTACTGGAG, 5' GATCCCGCTATTGTCTAGACCC, Tm 65°C; Y397/kinase Domain: 5' ACCCGGAGCTTGTGACAGACCTCAGTTATCATC, Tm 52°C; Prolinrich Region: 5' AGTCAGATATGCCTTACGCGGTGAGTACCG, 5' GTACACCTTATCATTGCACCGG, Tm 55°C; FAT Domain: 5' AAGGTGATCAGGAAATGTGACCGG, 5' CAGTTGTCGTCTGCTGCTCACCAGC, Tm 55°C. Each reaction (5 μl) was electrophoresed on a 1.5% agarose gel at 75 V for 1 h, and the resulting bands were excised and sequenced. As the conditions for RT-PCR were not designed to detect quantitative changes, the DNA in each RT-PCR product was quantitated, and equivalent ng of DNA from each sample were electrophoresed on a 1.5% agarose gel (Fig. 2).

Cells. The OFAK5 clone was described previously (26). The OFAK5 clone cells were harvested with buffered EDTA, resuspended in serum-free DMEM with 1% BSA or DMEM with 0.1% fetal bovine serum and 1% BSA and plated onto flasks coated with 5 μg/ml vitronectin for the indicated times.

RESULTS

FAK Protein in the Neoplastic Brain Fails to Undergo the Alternative Splicing Characteristic of FAK Processing in the Normal Adult Brain: FAK Protein in the Normal Adult Brain Migrates with a Slower Mobility. FAK protein in the adult rat brain migrates with a slower mobility (~129 kDa) because of alternative splicing in which inclusion of three short exons (Boxes 28, 6, and 7) are found around the autophosphorylation site (18). To determine whether FAK protein is spliced alternatively in astrocytic neoplasia, Western blot and RT-PCR analyses were performed on anaplastic astrocytoma and normal brain biopsy samples. SDS-PAGE analysis of 10 frozen biopsy samples from patients with grade III anaplastic astrocytoma and 10 frozen nonneoplastic brain biopsy samples from patients with a seizure disorder (5 samples), or from normal brain at autopsy (5 samples), indicated that the FAK protein in the tumor biopsy samples migrated with a relative molecular mass of 125 kDa, whereas the FAK protein in the nonneoplastic adult brain samples migrated with a relative molecular mass of 129 kDa (Fig. 1, A and B).

FAK protein Western blotted from two primary astrocyte preparations and from two malignant astrocytoma cell lines that had been propagated in media with fetal bovine serum migrated with the same relative mobility (125 kDa) as FAK protein in the tumor biopsies (Fig. 1, A and B). Proteolytic cleavage at the NH2 or COOH termini likely does not contribute to the observed difference in migration, as these Western blot analyses were performed with rabbit anti-NH2- and COOH-terminal FAK IgG directed toward residues 2–14 (Fig. 1A) and mAb anti-COOH-terminal FAK directed toward residues 900–1052 (Fig. 1B), and they demonstrate integrity of the FAK protein at the NH2 and COOH termini in all of the samples. Thus, degradation of the FAK...
The Levels of FAK Protein Are Elevated in Human Anaplastic Astrocytoma Biopsy Samples, and the FAK Protein Is Hyperphosphorylated. Our previous immunohistochemical analyses indicated that the levels of FAK protein are elevated in the tumor cells of malignant astrocytoma biopsies as compared with the levels in astrocytes and other glial cells of the normal brain (26). To confirm the increased levels of FAK protein and to determine the phosphorylation state of the FAK protein in these tumors in vivo, we performed Western blot analysis. Ten frozen biopsy samples from patients with grade III anaplastic astrocytoma and 10 frozen nonneoplastic brain biopsy samples from patients with a seizure disorder (5 samples), or from normal brain at autopsy (5 samples), were analyzed. A 2.5-fold estimated average increase in FAK protein expression was found in the grade III anaplastic astrocytoma biopsy samples (10 of 10; 5 samples shown in Fig. 3A, Lanes 6–10), as compared with the nonneoplastic brain biopsies (10 of 10; 5 samples shown in Fig. 3A, Lanes 1–5), when normalized to actin (Fig. 3D) using densitometric analysis. Furthermore, the FAK protein in the tumor biopsies exhibited a 4-fold estimated average increase in autophosphorylation (Y397) and a 10-fold estimated average increase in overall tyrosine phosphorylation, as compared with the nonneoplastic adult brain biopsy samples (Fig. 3, B and C), when normalized to total FAK using densitometric analysis. Longer exposure of the mAb PY20 blot revealed additional tyrosine phosphorylated proteins of various mobilities. All samples were also subjected to immunoprecipitation analysis with anti-FAK IgG, followed by Western blot with antiphosphotyrosine IgG, and the level of phosphorylation on tyrosine residues of this region revealed two bands, one of which (670 bp) encoded Boxes 6 and 7 (3 samples shown in Fig. 2, Lanes 3–5). The conditions for the RT-PCR were designed to detect qualitative differences in FAK mRNA size and were not designed to detect quantitative differences; thus, in Fig. 2, equivalent ng of DNA from each RT-PCR product were electrophoresed on the agarose gel. These data indicate that the relatively faster mobility of the FAK protein demonstrated on SDS-PAGE analysis of the nonneoplastic brain samples is because of a failure to undergo the alternative splicing typical of nonneoplastic adult brain.

Nonquantitative RT-PCR analysis followed by sequencing of the bands demonstrated distinct mRNA transcripts in the tumor and nonneoplastic brain biopsy samples for the region encoding the auto-phosphorylation site and part of the kinase domain of FAK. In the nonneoplastic adult brain biopsy samples (10 of 10), the RT-PCR for this region revealed two bands, one of which (670 bp) encoded Boxes 28, 6, and 7 and one of which (580 bp) encoded Boxes 6 and 7 (3 samples shown in Fig. 2B, Lanes 3–5). In contrast, in the anaplastic astrocytoma tumor biopsy samples (10 of 10), the RT-PCR analysis of this region revealed only one band (540 bp), which did not encode any of these three boxes (3 samples shown in Fig. 2B, Lanes 6–8). Moreover, similar analysis of primary adult human astrocytes (Fig. 2E, Lanes 3 and 4) and malignant astrocytoma cell lines (U-251 MG and U-87 MG; Fig. 2E, Lanes 5 and 6) that were propagated in culture revealed a single band equivalent to that found in the tumor biopsy samples. No differences in mRNA size or sequence were detected for the regions that encode the NH2 terminus, proline-rich region, or the FAT domain (Fig. 2, A, C, and D, respectively) in the anaplastic astrocytoma biopsy samples (3 samples shown in Fig. 2, Lanes 6–8) and the nonneoplastic brain biopsy samples (3 samples shown in Fig. 2, Lanes 3–5).
was estimated to be 10-fold higher in the tumor samples, as compared with the nonneoplastic brain samples (data not shown), similar to what we demonstrated in the Western blot analysis (Fig. 3C). These data demonstrate that the level of protein expression, autophosphorylation, and overall tyrosine phosphorylation of FAK is elevated in grade III anaplastic astrocytoma biopsy samples in vivo as compared with nonneoplastic adult brain samples.

Increased Src Kinase Activity Is Associated with FAK in Anaplastic Astrocytoma Biopsy Samples. To determine whether the enhanced phosphorylation of FAK on tyrosine residues in the anaplastic astrocytoma biopsy samples is because of increased Src kinase activity, the activation state of Src and its association with FAK was assessed. Three Src family members with relative molecular masses of 56, 60, and 62 kDa were detected in both the tumor and nonneoplastic adult brain biopsy samples. The levels of the Src family proteins were found to be lower in the tumor biopsy samples (10 of 10) than in the nonneoplastic adult brain biopsy samples (10 of 10; 7 samples of nonneoplastic brain and 8 samples of tumor shown in Fig. 4C), when normalized to actin (Fig. 4C). However, the tumor biopsy samples exhibited a 1.5–2-fold higher level of Src kinase activity, as compared with nonneoplastic adult brain samples, based on Western blot analysis using rabbit anti-Src (pY416) IgG, which detects active Src kinase (Fig. 4B). An in vitro Src kinase assay detecting Src phosphorylation of a synthetic substrate also demonstrated a 2-fold increase in Src kinase activity in 2 tumor samples, as compared with 2 normal brain samples, as well as a 2-fold increase in the Src kinase activity in U-251 MG and U-87 MG malignant astrocytoma cell lines, as compared with two preparations of primary human astrocytes (data not shown). Immunoprecipitation studies using anti-FAK or anti-Src IgG followed by Western blot analyses with anti-rabbit anti-FAK IgG, respectively, showed that the Src kinase is associated with the FAK protein in both the tumor and nonneoplastic adult brain biopsy samples (2 samples of each shown in Fig. 4D). However, the Src protein associated with FAK in anaplastic astrocytoma biopsy samples exhibited elevated levels of tyrosine phosphorylation, suggesting that the Src kinase associated with FAK is an active kinase (Fig. 4E). Stripping and reprobing with anti-Src (pY416) IgG revealed a band at 60 kDa only in the tumor samples (data not shown). The increased Src kinase activity in anaplastic astrocytoma biopsy samples is likely responsible, at least in part, for the increased phosphorylation of FAK on tyrosine residues in vivo.

Shc Coimmunoprecipitates with FAK Protein in Anaplastic Astrocytoma Biopsy Samples but not in Normal Adult Brain, and Shc Phosphorylation Is Enhanced in Tumor Biopsy Samples. Studies of cells propagated in culture indicate that phosphorylation of FAK at tyrosine 397 is necessary for the binding of Shc to FAK (7). The latter event is thought to result in the phosphorylation of Shc by FAK and Src (6, 7). Shc phosphorylation results in the recruitment of the Grb2/SOS complex and activation of Ras (30). To determine which forms of the Shc protein are expressed in tumors and whether

---

**Fig. 4.** Increased Src kinase activity in anaplastic astrocytoma biopsy samples that is associated with FAK. Human nonneoplastic brain biopsy samples (Lanes 1–7) and human anaplastic astrocytoma biopsy samples (Lanes 8–15) were detergent lysed, and equivalent μg of lysate from each sample were subjected to SDS-PAGE, transferred to Immobilon, immunoanalyzed, stripped, and reprobed, as described in “Materials and Methods.” A, blotted with mAb anti-c-Src; B, blotted with rabbit anti-Src (pY416) IgG; C, blotted with mAb antiax. Coimmunoprecipitation was performed using equivalent μg of lysate incubated with Sepharose-coupled antibodies, followed by SDS-PAGE, transfer to Immobilon, and immunoblot analysis, as described in “Materials and Methods.” D, immunoprecipitation with mAb anti-src, followed by blotting with rabbit anti-FAK IgG; E, immunoprecipitation with rabbit anti-FAK IgG, followed by blotting with mAb antiphosphotyrosine; F, the membrane in E was stripped and reprobed with mAb anti-Src.
Shc associates with FAK in the tumor samples, the level of Sch protein and phosphorylation, as well as its association with FAK, was examined. Two forms of Sch protein (46 and 52 kDa) were expressed in both the anaplastic astrocytoma biopsy samples (10 of 10) and in normal adult brain biopsy samples (10 of 10; 7 samples of each shown in Fig. 5A). However, the 52-kDa form of Shc was predominant in the tumor biopsy samples, in contrast to the nonneoplastic adult brain biopsy samples in which the 46-kDa form of Shc was predominant (Fig. 5A). Stripping and reprobing of the membrane with antiphosphotyrosine IgG demonstrated increased phosphorylation of the 52-kDa form of Shc in the tumor biopsy samples (Fig. 5B). Coimmunoprecipitation analyses indicated that Shc is associated with FAK in the anaplastic astrocytoma biopsy samples but not in the nonneoplastic brain biopsy samples (2 samples of each shown in Fig. 5D). Furthermore, the Sch protein associated with FAK in the tumor samples is phosphorylated (Fig. 5E). These data demonstrate differential expression of Sch protein isoforms in these tumors versus normal brain, increased phosphorylation of Shc in the tumors, and its association specifically with FAK in anaplastic astrocytoma biopsy samples.

To determine whether overexpression of FAK enhances Sch association and phosphorylation in vitro, stably transfected malignant astrocytoma cells with TET-inducible FAK (OFAK5 cells) were used. Administration of 0.1 μg/ml doxycycline for 4 days followed by harvesting, resuspending the cells in serum-free media with 1% BSA, and maintaining in suspension for 1 h or plated in serum-free media onto vitronectin-coated plates for 1 h, followed by detergent lysis. Equivalent μg of lysate were subjected to SDS-PAGE or immunoprecipitated with mAb anti-Shc and then Western blotted, as described in “Materials and Methods.” A. blotted with rabbit anti-FAK IgG; B. blotted with rabbit anti-FK (pY397); C. blotted with mAb anti-Shc; D. blotted with mAb antiphosphotyrosine; E. blotted with mAb antiaxin; F. immunoprecipitation with mAb anti-Shc, followed by blotting with rabbit anti-FAK IgG; in G, the membrane in F was stripped and reprobed with mAb anti-Shc.

**Fig. 6.** Overexpression of FAK in malignant astrocytoma cells in vitro increases Shc association and phosphorylation. OFAK 5 clones were administered 0.1 μg/ml doxycycline (+) or no doxycycline (−) for 4 days, harvested, resuspended in serum-free media with 1% BSA, and maintained in suspension for 1 h or plated in serum-free media onto vitronectin-coated plates for 1 h, followed by detergent lysis. Equivalent μg of lysate were subjected to SDS-PAGE or immunoprecipitated with mAb anti-Shc and then Western blotted, as described in “Materials and Methods.” A. blotted with rabbit anti-FAK IgG; B. blotted with rabbit anti-FK (pY397); C. blotted with mAb anti-Shc; D. blotted with mAb antiphosphotyrosine; E. blotted with mAb antiaxin; F. immunoprecipitation with mAb anti-Shc, followed by blotting with rabbit anti-FAK IgG; in G, the membrane in F was stripped and reprobed with mAb anti-Shc.

**Increased ERK-2 Activation and Elevated Levels of Cyclin D and E in Human Anaplastic Astrocytoma Biopsy Samples As Compared with Nonneoplastic Adult Brain Biopsy Samples.** To determine whether the increased phosphorylation of Shc in anaplastic astrocytoma biopsy samples results in increased activation of ERK-1 and -2, the formation of phasedo-ERK-1 and -2 was evaluated by Western blot analysis. In the anaplastic astrocytoma biopsy samples (10 of 10), a >5-fold increase in activated ERK-2
was found, as compared with nonneoplastic brain biopsy samples (5 samples of each shown in Fig. 7A). Stripping and reprobing with antitotal ERK IgG revealed equivalent levels of total ERK protein in the tumor and nonneoplastic brain biopsy samples (Fig. 7B). As FAK has been shown to signal to JNK kinase in fibroblasts in vitro (31), we also examined JNK activation. In contrast to our findings concerning ERK, lower levels of activated JNK (phospho-JNK) were found in the anaplastic astrocytoma biopsy samples (10 of 10) than in the nonneoplastic brain biopsy samples (5 samples of each shown in Fig. 7C).

Zhao et al. (32) have reported recently that overexpression of FAK in fibroblasts promotes ERK activation and cell cycle progression. To determine whether the increased activation of ERK-2 in anaplastic astrocytoma biopsy samples correlated with cell cycle progression, Western blot analysis for cyclins D and E was performed. Elevated levels of cyclin D and E were found in the nonneoplastic adult brain biopsy samples (10 of 10), as compared with nonneoplastic adult brain biopsy samples (10 of 10; 5 samples of each shown in Fig. 8, A and C), when normalized to actin (Fig. 8, B and D).

To determine whether overexpression of FAK enhances ERK activity in vitro, the OFAK5 cells were used. OFAK5 cells administered 0.1 μg/ml doxycycline for 4 days, followed by plating onto vitronectin in media with 0.1% fetal bovine serum, and 1% BSA for 24 h demonstrated sustained phosphorylation of tyrosine 397 (Fig. 9B), as well as elevated ERK-2 activity (Fig. 9, C and D). No difference in ERK activity was observed between the induced and uninduced OFAK5 clone during initial attachment to vitronectin (30 min and 1 h; data not shown), suggesting that early anchorage-dependent signals to ERK are not modulated by FAK overexpression in these cells under the conditions tested.

FAK Protein Is Not Phosphorylated in Grade I Astrocytic Tumors. To determine whether the activation/phosphorylation state of FAK correlates with the grade of the astrocytoma, Western blot analysis of grade I astrocytic tumor biopsies (pilocytic astrocytomas)

Fig. 7. Elevated ERK-2 activity in human anaplastic astrocytoma biopsy samples. Human nonneoplastic brain biopsy samples (Lanes 1–5) and human anaplastic astrocytoma biopsy samples (Lanes 6–11) were detergent lysed, and equivalent μg of lysate were subjected to SDS-PAGE, transferred to Immobilon, immunoblotted, stripped, and reprobed, as described in “Materials and Methods.” A, blotted with rabbit anti-FAK IgG; B, blotted with rabbit anti-ERK IgG; C, blotted with mAb antiphospho-JNK; D, blotted with mAb anti-JNK.

Fig. 8. Elevated levels of cyclin D and E in human anaplastic astrocytoma biopsy samples. Human nonneoplastic brain biopsy samples (Lanes 1–5) and human anaplastic astrocytoma biopsy samples (Lanes 6–10) were detergent lysed, and equivalent μg of lysate were subjected to SDS-PAGE, transferred to Immobilon, immunoblotted, stripped, and reprobed, as described in “Materials and Methods.” A, blotted with mAb anticyclin D; B, blotted with mAb anticyclin E; C, blotted with mAb antiphospho-ERK IgG; D, blotted with mAb anti-ERK IgG; E, blotted with mAb antiactin.

Fig. 9. Overexpression of FAK increases ERK-2 activity in malignant astrocytoma cells. OFAK5 cells were administered 0.1 μg/ml doxycycline (+) or no doxycycline (−) for 4 days, harvested, and plated onto vitronectin-coated plates in DMEM with 0.1% fetal bovine serum and 1% BSA for 24 h. Cells were then detergent lysed, and equivalent μg of lysate were subjected to SDS-PAGE and Western blotted, as described in “Materials and Methods.” A, blotted with rabbit anti-FAK IgG; B, blotted with rabbit anti-FAK (pY397); C, blotted with rabbit anti-phospho-ERK IgG; D, blotted with rabbit anti-ERK IgG; E, blotted with mAb antiphospho-JNK. was performed. Grade I astrocytomas have a ~90% 5-year survival if the tumor is excised completely. In the grade I astrocytic tumor biopsy samples tested (3 of 3), we found lower levels of FAK protein and tyrosine phosphorylation than in the anaplastic astrocytoma biopsy samples (3 of 3; Fig. 10, A and B). Moreover, the grade I astrocytoma biopsies exhibited lower levels of ERK-2 activation (Fig. 10, D and E), suggesting that increased FAK activity correlates with increased ERK-2 activity in astrocytic tumors.

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2002 American Association for Cancer Research.
DISCUSSION

To determine the role of endogenous FAK in the promotion of the proliferative phenotype of malignant astrocytes, we investigated the activity and downstream signaling events of FAK in human anaplastic astrocytoma biopsy samples. This approach provides insights into endogenous signaling as it occurs in a disease state in vivo and avoids the limitations on the interpretation of signaling events that are altered by in vitro culture.

We found that the FAK protein in human anaplastic astrocytoma biopsy samples migrated with a faster mobility on SDS-PAGE than FAK protein from nonneoplastic adult brain biopsy samples. This faster mobility is similar to that reported by other investigators for biopsy samples. The limitations on the interpretation of signaling events that are altered endogenous signaling as it occurs in a disease state astrocytoma biopsy samples. This approach provides insights into FAK SIGNALING IN TUMORS IN VIVO

FAK is known to be activated by integrin engagement and growth factor stimulation (6). Consistent with these observations, anaplastic astrocytoma tumor cells have been shown to exhibit up-regulation of several integrin and growth factor receptors in vivo, which likely contributes to the increased FAK activity (25, 36). The elevated overall tyrosine phosphorylation of FAK that we show in the tumor biopsy samples suggests other tyrosine residues (such as Y576, Y577, Y861, and Y925) are also phosphorylated on tyrosine residues in Src-transformed fibroblasts (38). As Src can phosphorylate FAK at tyrosine 397 (39), our finding of increased FAK phosphorylation in tumors versus normal brain suggests that, in neoplasia, FAK phosphorylation may recapitulate ontogeny.

To determine whether Src kinase contributes to the increased FAK phosphorylation found in anaplastic astrocytoma biopsy samples, we examined the level of Src kinase activity in the tumor and normal brain samples and found elevated Src kinase activity in the tumor biopsy samples. This is likely attributable, at least in part, to the up-regulation of tyrosine kinase growth factor receptors in these tumors (37). Other investigators have reported that FAK is heavily phosphorylated on tyrosine residues in Src-transformed fibroblasts (38). As Src can phosphorylate FAK at tyrosine 397 (39), our finding of increased Src kinase activity provides another mechanism for the elevated tyrosine 397 phosphorylation of FAK in our tumor biopsy samples. We also demonstrate a FAK/Src complex in the tumor and the normal brain biopsy samples; however, in the tumor samples, the pool of Src associated with FAK exhibits increased phosphorylation on tyrosine residues. This suggests the possibility of downstream signals from the FAK/Src complex in these tumors. Src kinase may play a role in the transformation of astrocytes, as the v-Src transgenic mouse with v-Src under the control of an astrocyte-specific promoter (GFAP) results in the neoplastic transformation of astrocytes in histopathological analysis, a marked increase in astrocytes, and a severe loss of neurons, the RT-PCR analysis demonstrated alternative splicing of FAK mRNA, and the Western blot analysis demonstrated FAK protein to migrate with a slower mobility (129 kDa). The different results found when analyzing the normal astrocytes propagated in vitro versus the results found when analyzing the nonneoplastic brain could be because of several possibilities, including differences in FAK splicing when astrocytes are propagated in vitro versus in vivo and, thus, the necessity for in vivo studies or very low expression of the FAK gene in nonneoplastic astrocytes in vivo. The absence of these alternatively spliced FAK mRNAs may be a useful diagnostic marker for malignant astrocytoma.

Our Western blot analyses of FAK protein demonstrate a consistent increase in FAK protein expression, autophosphorylation, and overall tyrosine phosphorylation in tumor biopsy samples, as compared with nonneoplastic brain biopsy samples. The 2.5-fold estimated average increase in FAK protein expression that we demonstrate confirms our previous immunohistochemical analysis indicating elevated levels of FAK protein in malignant astrocytoma cells as compared with astrocytes in normal brain (26). Other investigators also have reported elevated levels of FAK expression in tumors, largely through the use of immunohistochemical analyses (20–23). Agochiya et al. (34) recently reported elevated levels of FAK protein in several human cancer cell lines and showed amplification of the FAK gene locus in these cell lines. This amplification occurred as an en-bloc amplification of a region of chromosome 8 and included the c-myc gene, which is adjacent to the fak gene (34). The c-myc gene is amplified in malignant astrocytic tumors (35); thus, amplification of the fak gene in malignant astrocytomas potentially could contribute to the elevated FAK protein expression. Our finding of increased FAK phosphorylation at tyrosine 397 suggests enhanced FAK activity in anaplastic astrocytoma tumor biopsy samples. FAK is known to be activated by integrin engagement and growth factor stimulation (6). Consistent with these observations, anaplastic astrocytoma tumor cells have been shown to exhibit up-regulation of several integrin and growth factor receptors in vivo, which likely contributes to the increased FAK activity (25, 36). The elevated overall tyrosine phosphorylation of FAK that we show in the tumor biopsy samples suggests other tyrosine residues (such as Y576, Y577, Y861, and Y925) are also phosphorylated. Other investigators have reported increased FAK tyrosine phosphorylation in embryonic versus adult rat brain (19); thus, our findings of increased FAK activity and overall tyrosine phosphorylation in tumors versus normal brain suggests that, in neoplasia, FAK phosphorylation may recapitulate ontogeny.

To determine whether Src kinase contributes to the increased FAK phosphorylation found in anaplastic astrocytoma biopsy samples, we examined the level of Src kinase activity in the tumor and normal brain samples and found elevated Src kinase activity in the tumor biopsy samples. This is likely attributable, at least in part, to the up-regulation of tyrosine kinase growth factor receptors in these tumors (37). Other investigators have reported that FAK is heavily phosphorylated on tyrosine residues in Src-transformed fibroblasts (38). As Src can phosphorylate FAK at tyrosine 397 (39), our finding of increased Src kinase activity provides another mechanism for the elevated tyrosine 397 phosphorylation of FAK in our tumor biopsy samples. We also demonstrate a FAK/Src complex in the tumor and the normal brain biopsy samples; however, in the tumor samples, the pool of Src associated with FAK exhibits increased phosphorylation on tyrosine residues. This suggests the possibility of downstream signals from the FAK/Src complex in these tumors. Src kinase may play a role in the transformation of astrocytes, as the v-Src transgenic mouse with v-Src under the control of an astrocyte-specific promoter (GFAP) results in the neoplastic transformation of astrocytes in
FAK SIGNALING IN TUMORS IN VIVO

In the normal brain, Src could potentially associate with FAK in a phosphorylation-independent manner, as Src formed complexes with FAK in both the nonneoplastic brain and in the tumor biopsy samples. A recent report from Hauck et al. (41) indicates that v-Src can interact with FAK via its SH3 domain, providing one possible explanation for Src association with FAK in the nonneoplastic brain. In addition, the minimal phosphorylation of FAK at tyrosine 397 in the nonneoplastic brain biopsy samples could be attributable, at least in part, to expression of the alternative splice variants around the autophosphorylation site (Boxes 6 and 7), which can inhibit Src’s ability to phosphorylate FAK at tyrosine 397 in COS cells (33), as described above.

In investigating these tumor biopsy samples for evidence of downstream signaling that could be linked to FAK activity, we found elevated expression and phosphorylation of the 52-kDa isoform of Shc in the anaplastic astrocytoma biopsy samples. In the nonneoplastic brain biopsy samples, we found predominant expression of the 46-kDa Shc isoform and in the tumor biopsy samples, predominant expression of the 52-kDa Shc isoform. This novel observation of a shift in the predominant Shc isoform with neoplasia could have biological relevance; however, at present, its meaning is unclear, as the homology in sequence, domains, and known functions of these two Shc isoforms is high (30). These studies also revealed a unique association of phosphorylated Shc and FAK in the tumors, as compared with the normal brain, suggesting that FAK could be promoting Shc phosphorylation and subsequent Ras activity in these tumors. This is supported by in vitro data in which we show that overexpression of FAK in malignant astrocytoma cells results in increased FAK activity and Shc association with FAK. Elevated Shc phosphorylation in malignant astrocytoma cell lines has been reported (28), and anaplastic astrocytoma biopsy samples have been reported to exhibit enhanced Ras activity as compared with normal brain that is independent of Ras gain-of-function mutations (27). However, the upstream activators of Ras in these tumors have not been defined clearly. Phosphorylation of the 52- and 46-kDa forms of Shc at tyrosine 317 has been shown to be necessary for Grb2/SOS binding and subsequent Ras activation (30), and Schlaeffer et al. (7) have reported that FAK autophosphorylation leads to Shc binding to FAK and FAK phosphorylation of Shc at tyrosine 317. Shc may also be phosphorylated in the anaplastic astrocytoma tumor biopsy samples by a FAK-independent mechanism, such as by integrin engagement and subsequent activation of Src or Fyn via a caveolin-dependent mechanism (42). Nevertheless, our data suggest that activated FAK in these tumor biopsy samples most likely phosphorylates Shc, thereby promoting elevated Ras activity.

Shc phosphorylation and subsequent Ras activation leads typically to activation of ERK (30). We found higher ERK activity in the tumor biopsy samples than in the nonneoplastic brain biopsy samples. The latter is likely secondary to increased FAK activity, at least in part, and this is supported by the work of other investigators (5, 7, 43). Moreover, grade I astrocytic tumor biopsy samples, which exhibit minimal levels of FAK activity, exhibit lower levels of ERK activity. In support of our finding in the anaplastic astrocytoma biopsy samples, we also show that overexpression of FAK in malignant astrocytoma cells in vitro resulted in sustained ERK-2 activity. We reported previously that overexpression of FAK in these cells resulted in increased soft agar growth and haptotactic migration (26). Tyrosine kinase growth factor receptors (e.g., platelet-derived growth factor-receptor and epidermal growth factor-receptor) and integrin-mediated pathways that can activate ERK independent of FAK, such as the \( \alpha_B \beta_x \) activation of ERK through the caveolin/Src or Fyn complex, may also play a role in activating ERK in these tumors in vivo. Other investigators have reported JNK to be activated in FAK-mediated cell cycle regulation in cell lines propagated in vitro (31). We did not detect elevated JNK activity in the anaplastic astrocytoma biopsy samples, suggesting that the elevated FAK activity in these tumors is not signaling to JNK kinase.

The elevated levels of cyclin D and E that we detect in these tumors are consistent with the unregulated cell proliferation found in vitro and with a role for ERK in promoting this cell proliferation. Other investigators have reported that exogenous expression of FAK in fibroblasts leads to elevated cyclin D expression and acceleration of the G1 to S phase transition of the cell cycle (10, 32). Consistent with our Western blot analysis, other investigators have reported elevated cyclin D expression in grade III and IV astrocytomas (44), and malignant astrocytoma cell lines have been reported to demonstrate aberrant cyclin E expression (45).

In summary, our data suggest that the elevated endogenous FAK expression and activity in anaplastic astrocytoma biopsy samples plays a role in promoting the unregulated proliferation of these tumors in vitro through FAK phosphorylation of Shc, subsequent activation of ERK, and a resultant increase in cyclin D and E expression. Our novel observation that the levels of Src kinase activity are enhanced and that phosphorylated Src kinase is associated with FAK in these tumor biopsy samples provides a mechanism for the increased overall FAK phosphorylation on tyrosine residues. As elevated levels of FAK protein have been reported in multiple malignant tumors, we hypothesize that the elevated levels of FAK protein, along with enhanced tyrosine kinase growth factor receptor activation, leads to increased activation of FAK and potentially Src, followed by an increase in Src phosphorylation, ERK activation, and subsequent tumor cell proliferation.

ACKNOWLEDGMENTS

We thank Suzanne Perry for secretarial assistance and Dr. Wen-Cheng Xiong (University of Alabama at Birmingham) for reading this manuscript.

REFERENCES

FAK SIGNALING IN TUMORS IN VIVO


Focal Adhesion Kinase Enhances Signaling through the Shc/Extracellular Signal-regulated Kinase Pathway in Anaplastic Astrocytoma Tumor Biopsy Samples


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/9/2699

This article cites 43 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/9/2699.full.html#ref-list-1

This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/62/9/2699.full.html#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.