Overexpression of the Focal Adhesion Kinase (p125FAK) in Invasive Human Tumors


Abstract

The focal adhesion kinase (FAK) gene encodes a tyrosine kinase (p125FAK) thought to be involved in signal transduction pathways used in cell adhesion, motility, and anchorage-independent growth. Because alterations in these cellular processes occur in tumor invasion and metastasis, we studied the protein expression of FAK in a variety of human tumors and found that in the 119 samples studied, increased levels of p125FAK correlated with the invasive potential of a tumor. By comparing FAK expression in tumors with normal tissue from the same patient, we found that p125FAK was significantly elevated in 17 (100%) of 17 invasive and metastatic colon lesions and in 22 (88%) of 25 invasive and metastatic breast tumors. Additional studies of FAK expression in 13 high grade sarcomas showed high levels in all samples compared to benign, noninvasive mesenchymal specimens. Furthermore, FAK protein levels were elevated in preinvasive lesions, such as large (>2 cm) colonic villous adenomas, whereas noninvasive, yet hypercellular, neoplastic tissues such as parathyroid and hepatocellular adenomas did not overexpress FAK. These data provide evidence that both epithelial and mesenchymal tumor progression are accompanied by increased p125FAK expression and suggest that the level of FAK expression might be a marker for the invasive potential of a tumor.

Introduction

The invasion of cancer is a complex process that includes alterations in cell adhesion and motility, allowing tumor cells to attach to and migrate through the extracellular matrix and invade the underlying connective tissue (1). Some of these alterations develop at cell/ECM contact points known as focal adhesions, which consist of membrane-associated and cytoskeletal components, ECM proteins, as well as intracellular signaling molecules. One of these putative signaling molecules is a protein tyrosine kinase called FAK (2, 3), a Mr 125,000 protein (p125FAK) that becomes phosphorylated when integrins are clustered with mAbs (4) or when cells adhere to the ECM (5). FAK also becomes phosphorylated when cells are stimulated by mitogenic neuropeptides such as bombesin (6) or when transformed by v-src (7). Although there is no evidence of somatic mutations of the FAK gene in human tumors or that p125FAK has transforming potential, recent studies indicate pp60src forms a stable association with the tyrosine phosphorylated form of FAK through the Src SH2 domain, suggesting FAK plays a role in signal transduction (8). The convergence of these various properties at p125FAK suggests that this protein is used in a variety of cellular processes, regulating a diverse set of normal and abnormal functions, such as cell adhesion, motility, and ultimately, proliferation.

We have studied previously the expression of FAK mRNA in a small group of human tissues and have shown that increased levels of FAK accompany changes in epithelial and mesenchymal tumors during their progression to an invasive phenotype (9). In this study, we have produced a specific antibody to recombinant p125FAK and studied the levels of FAK protein expression in a variety of cell lines and 119 different human tissue specimens to determine whether increased levels of p125FAK are associated with malignant disease. The results reported here include a diverse collection of neoplastic tissues and confirm our preliminary observations that the overexpression of FAK correlates with the acquisition of invasive potential by tumor cells, as well as indicate that FAK expression levels may be useful as a marker of occult invasion in premalignant conditions.

Materials and Methods

Cell Lines. Human cell lines were obtained from the American Type Culture Collection (Rockville, MD), except for the NHF and human embryonal RD cell lines, which were kindly gifts from Drs. Yue Xiong and Bernard Weissman, respectively.

Tissue Samples. Tissue samples were collected from 72 patients at the University of North Carolina Hospitals from operative specimens banked through Institutional Review Board-approved protocols. The tissue banking was performed by the Lineberger Comprehensive Cancer Center Tumor Procurement Facility and the University of North Carolina-Specialized Program of Research Excellence Tissue Procurement and Analysis Core Facility. Normal colonic mucosa, colon adenocarcinoma, and sarcoma tissues were dissected from underlying connective tissue. Other tissue samples were separated from the surrounding normal parenchyma by sharp dissection. Histopathological tissue confirmation was performed on all tissue samples by a reference pathologist (G. D.). After dissection of the samples was completed, the tissues were snap frozen in liquid nitrogen and stored at −70°C.

Antibody Preparation. In the process of screening human tumor tissue for novel tyrosine kinases, we cloned a 180-bp fragment of human FAK (10). This fragment was used to screen a BT20 breast cancer library and a full-length clone of human FAK was isolated. For the preparation of a FAK-specific polyclonal antibody, a PCR fragment encoding a region of the 5' end of our FAK clone was subcloned into the HindIII site of the pQE30 plasmid (Qiagen, Chatsworth, CA), forming a fusion protein of six histidine residues and amino acids 248–314 of the FAK protein. This fragment did not include the portion of the FAK gene encoding the COOH-terminal, FAK-related nonkinase (11). The protein was purified according to the manufacturer's specifications and purified over a Ni2+-NTA resin column. To "renature" the peptide, a step gradient of 8-0 M urea was used in renaturation buffer [50 mm sodium bicarbonate (pH 8.5), 500 mm NaCl, and 20% glycerol], followed by an extensive wash with renaturation buffer. The resulting M, 6000 recombinant peptide was used for polyclonal antibody production in New Zealand White rabbits.

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4 The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; NHF, normal human fibroblast; RD, rhabdomyosarcoma.
Protein Extraction and Western Blot Analysis. For the preparation of RD cell lystate, cells were washed once with cold PBS, then overlayed with NP-40 lysis buffer [1% NP-40, 20 mm Tris (pH 7.4), 150 mm NaCl, 5 mm EDTA, 1 mm NaN₃, 10 µg/ml of the protease inhibitors aprotinin and leupeptin] on ice for 10 min and then harvested. The samples were clarified by centrifugation at 12,000 rpm for 10 min at 4°C, and the protein concentration was measured by the BCA assay (Pierce Chemical Co., Rockford, IL). Human tissues were extracted in 1-cm² sections in the same buffer but were disrupted with a Polytron PT 1200 (Brinkman Instruments, Inc., Westbury, NY) for 30 s on the maximum setting. For Western blot analysis, samples were analyzed by SDS-PAGE (50 µg total protein/lane) on 10% gels and blotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) as described (13). The V39 and ab1 (19—110; Santa Cruz, Santa Cruz, CA) antibodies were used for Western blotting at 1:1000 and 1:100 dilutions, respectively. Protein was visualized by the enhanced chemiluminescence detection system (Amsersham, Arlington Heights, IL).

Analysis of Data. The level of FAK protein expression in individual tumors was determined by densitometric scanning of the M, 125,000 band on autoradiographs of the Western immunoblots. Densitometry was performed with the use of a MTI CCD 72 spectrophotometer (Dage-MTI, Inc., Michigan City, IN), and FAK expression levels were normalized based on FAK protein expression levels in RD cells, a positive control. Subsequent data were expressed as a percentage increase relative to normal tissue for the particular tumors and presented as a scattergram plot. Statistical significance between neoplastic groups versus normal tissues was determined by the Student t test.

Results

p125FAK Is Overexpressed in Tumor Cell Lines. To characterize our anti-FAK antibody, V39, we studied the expression levels of FAK in a variety of human cell lines (Fig. 1). V39 specificity was demonstrated by Western blot detection of a M, 125,000 protein with no p125FAK signal detected with preimmune serum (Fig. 1) or with antibody preincubated with immunogenic peptides (data not shown). Low levels of p125FAK expression were seen in normal cells derived from mesenchymal tissue such as NHF and WI-38 cell lines. In contrast, high levels of FAK expression were seen in a variety of both epithelial and mesenchymal tumor cell lines. These included transformed cells derived from colon (LS180, LS174T, and COLO205) and breast cancers (BT474 and BT-20) as well as sarcomas (RD). These expression results suggested a correlation between increased levels of FAK protein in human cells with a malignant phenotype.

125FAK Is Overexpressed in Invasive Epithelial Tumors. To extend our findings of p125FAK overexpression in transformed cell lines, we studied FAK expression in human tissues derived from epithelial cells. We initially focused on colon and breast tissues as these provided an array of normal, neoplastic noninvasive, and malignant tissue types, as well as the opportunity to examine paired samples of tissues from individual patients. In colon samples, we found that p125FAK was expressed at minimally detectable levels in normal colonic mucosa and in small adenomatous polyps, which have essentially no invasive potential (Table 1). In contrast, seven large (>2 cm), premalignant villous adenomas and six primary, invasive adenocarcinomas had significantly elevated levels of p125FAK. This result was most noticeable in the matched samples of normal colon, invasive colonic adenocarcinoma, and metastatic carcinoma from the same patient where the highest levels of p125FAK protein were observed in the invasive and metastatic lesions compared to the normal colonic epithelium (Fig. 2A).

We found similar results in the breast tissues. Only 1 of the 23 paired normal and malignant samples showed any elevation of p125FAK in the normal breast ductal epithelial tissue, whereas 19 of the 22 (86%) invasive ductal adenocarcinoma specimens exhibited significant increases in FAK expression (Table 1). Similarly, high levels of p125FAK protein expression were seen in metastatic axillary lymph node samples (Fig. 2B).

To ensure that these findings were tumor specific and not an artifact of hypoeucellularity in the normal tissue specimens, we studied parathyroid and hepatocellular adenomas for their levels of p125FAK expression (Table 1). These epithelial lesions are neoplastic and hypercellular but have no invasive or metastatic potential and resulted in minimally detectable levels of p125FAK (Fig. 2B). Furthermore, because invasive tumors are known for their vascularity and occasional inflammatory infiltrates, we examined FAK expression from tissues derived from cavernous hemangiomas and peripheral blood lymphocytes. Once again, p125FAK levels were significantly lower compared to the levels seen in malignant tissues (data not shown). From these results it appeared that elevated levels of p125FAK were associated only with tumor cells that were in the process of becoming invasive or had already demonstrated their capacity for invasion and metastasis.

p125FAK Is Overexpressed in Invasive Mesenchymal Tumors. Next, we studied the levels of p125FAK expression in mesenchymal tumors to determine whether overexpression of FAK might be a common finding in tumors from different cellular origins. We examined both high-grade soft tissue sarcomas, such as malignant fibrous histiocytomas and leiomyosarcomas, as well as benign, noninvasive,
mesenchymal tumors, such as lipomas, leiomyomas, and fibroadenomas (Fig. 2C). All 13 high grade sarcoma tumors studied showed high levels of p125FAK expression. However, compared to invasive sarcomas, the noninvasive mesenchymal tumors failed to show significant elevations in FAK expression, despite their hypercellularity and large size (Table 1). Thus, mesenchymal tumors appeared to have a similar paradigm of p125FAK expression as the epithelial tumors, with overexpression of FAK in invasive tumors but low expression in the neoplastic lesions, which, despite a large size, had no potential for invasion and metastasis.

**Densitometric Analysis of the Relative Levels of p125FAK Expression in Human Tissue Samples.** To further study the changes in levels of p125FAK expression with invasion and metastasis, we consolidated the FAK protein expression results of all our specimens into a single format. After semiquantitation of the FAK levels by video densitometry and after obtaining the ratio of FAK expression in each tissue sample to the positive RD control sample on each blot, we determined the relative levels of p125FAK expression in all 119 human tissues. These are presented in a scattergram plot (Fig. 3). On the basis of the pathological characteristics of the tumor relative to normal tissue, increased levels of p125FAK expression were associated with the invasive and metastatic phenotypes compared to normal and neoplastic tissues without invasive potential.

![Fig. 2. Representative Western blots of p125FAK expression in human epithelial and mesenchymal tumors.](image)

![Fig. 3. Scattergram plot of relative p125FAK expression levels at different stages of malignant progression in the 119 human tissue specimens studied.](image)

**Discussion**

The processes of invasion and metastasis are late events in tumor progression, whereby tumor cells acquire the ability to migrate through the extracellular matrix and establish anchorage-independent growth in other organs. These events occur in a series of discrete steps beginning with tumor cell adhesion to extracellular matrix determinants in the host basement membrane (14, 15). The subsequent signaling events that allow the tumor cell to transverse the extracellular matrix are incompletely understood but appear to be centered at contact points between the cell and ECM, where signaling molecules such as integrins, FAK, and src are localized. Our results have demonstrated that the capacity for invasion and metastasis of transformed cells is accompanied by overexpression of p125FAK. Thus, it is possible that overexpression of p125FAK may be a common pathway for a variety of epithelial and mesenchymal tumor types to gain invasive potential.

The significance of these findings is further underscored by our results in the neoplastic tissues that were not invasive. We did not detect significantly elevated levels of p125FAK in any of the epithelial or mesenchymal neoplasms studied, which, although large and hypercellular, had no clinical or pathological evidence of invasion or metastasis. Furthermore, we found minimal levels of FAK in normal mesenchymal cells, as well as lymphocytes and benign vascular lesions, providing additional evidence that increased p125FAK expression is specific to malignant tumors. In contrast to these benign lesions, we found that FAK was overexpressed in large (>2 cm)
colonic villous adenomas that were treated by radical surgical resections. These lesions, although rare, are recognized for their high invasive potential with over 50% being malignant at the time of excision (16). These results, taken together with the progressive increases in the levels of FAK expression present in invasive and metastatic tumors of all types, suggest that FAK may be clinically useful as a marker of early cellular events leading to invasion.

It is intriguing to postulate why p125FAK might become overexpressed as part of the invasive and metastatic process. FAK is a normal gene with detectable levels of mRNA in all adult tissues (17) and no evidence for mutations, which would render it a transforming gene (18). In normal cells, FAK might be a sensor of cell adhesion, limiting growth in an anchorage-dependent manner, whereas in transformed cells, overexpression of FAK may override this regulation and allow anchorage-independent growth in the absence of cell adhesion. Studies of other tyrosine kinases such as c-src in human malignancies have shown that the overexpression of a tyrosine kinase results in increased specific enzymatic activity (19). In these studies, we have not examined the phosphorylation status of p125FAK because our antibody is poorly suited for immunoprecipitation assays. However, we can demonstrate weakly detectable autophosphorylation of p125FAK in tumors with high levels of FAK expression by immune complex kinase assays (data not shown), suggesting that the overexpressed protein does retain its intrinsic kinase activity similar to studies on other tyrosine kinases (20).

Finally, these observations raise the possibility that FAK might be a rational therapeutic target to interrupt the invasive and metastatic process. Because we have reproducibly demonstrated minimally detectable p125FAK levels in normal cells and high levels of p125FAK in invasive cancers, it would be useful to study the effects of attenuating FAK expression or interrupting its signal transduction pathway in tumor cells. If this causes the loss of anchorage-independent growth properties of a tumor, molecular-based therapeutics against FAK may become useful in the treatment of invasive and metastatic cancer.

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