Decreased Focal Adhesion Kinase Suppresses Papilloma Formation during Experimental Mouse Skin Carcinogenesis

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Abstract

Although focal adhesion kinase (FAK) is elevated in epithelial cancers, it is not known whether FAK expression influences tumor development in vivo. We found that fak +/- heterozygous mice display reduced 7,12-dimethylbenz[a]anthracene-induced papilloma formation that correlates with reduced FAK protein expression in the skin. However, the frequency of malignant conversion of papillomas into carcinomas is indistinguishable in fak +/- mice and their wild-type fak +/- littermates, most likely because papilloma FAK protein expression is elevated to wild-type levels. We also found that keratinocyte FAK protein expression is important for cellular responses downstream of ras in vitro (monitored by extracellular signal-regulated kinase activation after integrin engagement). Because 7,12-dimethylbenz[a]anthracene induces an activating mutation of H-ras, this provides one possible explanation for suppression of papilloma formation when FAK protein is limiting.

Introduction

Changes to the composition and function of cell-ECM adhesions can alter the cell’s capacity to attach and migrate through surrounding tissue. It is therefore likely that changes in expression and activities of the components of such adhesions could make an important contribution to cancer development (1). At the cellular level, focal adhesion components control various important aspects of oncogenic behavior, including anchorage-independent survival and growth (2), cytoskeletal disorganization (3), and altered adherence to ECM (4).

FAK is a nonreceptor tyrosine kinase, specifically located at cell-matrix adhesions, that acts at a point of convergence in a variety of signaling pathways known to influence cell adhesion and structure, motility, growth, survival, and differentiation (5). Elevated expression of FAK protein is also associated with a wide variety of human epithelial cancers. Work from our laboratory has demonstrated that FAK protein is elevated in cell lines derived from squamous cell carcinomas of the head and neck when these were compared with normal keratinocytes (6) and also during the malignant conversion of colonic adenoma cells to their carcinoma counterparts in vitro (7). Other studies have found similar elevated FAK protein expression in cervical carcinoma cell lines (8), prostatic carcinoma tumors (9), and colon and breast tumors and cell lines, where a correlation between FAK protein expression and the invasive potential of tumors has also been described (10). Despite this mounting circumstantial evidence supporting the link between FAK and the malignant phenotype, it has not been established whether FAK plays a direct role during tumorigenesis in vivo. In this study, we used a gene targeting approach to examine the effects of modulating the in vivo expression levels of FAK, in association with the well-characterized mouse skin carcinogenesis system (11). Specifically, we examined the effect of reduced FAK protein expression in the skin during tumor formation and progression in vivo. The mouse skin model of multistage carcinogenesis is well understood with respect to both the phenotypic and genetic changes that occur (12, 13). Because homozygous deletion of the fak gene is embryonically lethal (14), we used mice containing a heterozygous fak deletion. These mice exhibited a corresponding reduction in FAK protein expression in the epidermis and a significant reduction in the formation of benign skin tumors (papillomas).

Materials and Methods

Animals. Mice carrying a targeted mutation in the fak gene were generated by insertion of a neomycin cassette into the first exon of the FAK kinase domain. A 1161-bp cDNA fragment encoding murine fak nucleotides 550-1711 (15) was used as a probe to screen a murine 129Sv genomic library (kindly provided by H. Baribault, Burnham Institute, La Jolla, CA). A targeting vector was constructed using a 1.8-kb fragment containing the first exon of the kinase domain (nucleotides 1344–1441; Ref. 15), flanked by 2.1-kb (5') and 1.8-kb (3') homology arms. Insertion of the neomycin cassette results in gene disruption and causes a frameshift such that no functional mRNA is produced. The targeting vector was linearized and introduced into embryonic stem cells by electroporation. G418-resistant clones were isolated, and homologous recombination was verified by Southern blotting using internal and external probes. Targeted clones were injected into C57BL/6 blastocysts (The Burnham Institute Transgenic Facility), and resulting chimeric males crossed with C57BL/6 females to obtain germ-line transmission. All mice used were then crossed through four generations into an FVB inbred genetic background; control mice were homozygous for wt fak (fak +/-) and were littermates taken from the same breeding colony. Mice containing a heterozygous deletion in the fak gene were identified by PCR analysis of tail DNA using the following primers: F1 (5'-gcc att gaa gat gga ttg cac-3') and B1 (5'-gct cgg ctt cag c-3').

Chemical Carcinogenesis. Chemical carcinogenesis was performed on 40 8-week-old female mice. Animals were split into two groups, one containing 20 (+/−) fak mice and the other containing 20 (fak +/+) fak control animals. Both groups were treated in an identical manner throughout. Tumors were induced in vivo by shaving the dorsal skins of the mice, and 2 days later, topically applying a single dose of 25 μg of DMBA in 150-μl acetone. Mice were then treated topically twice a week for 20 weeks with 6.25 μg of TPA in 150-μl acetone. The number of benign and malignant tumors was recorded weekly for 45 weeks after DMBA treatment. Benign tumor numbers did not increase after 24 weeks attributable to the discontinuation of TPA treatment. Tumor tissue was snap frozen in liquid nitrogen and/or fixed overnight in
phosphate buffered formalin, followed by paraffin embedding. Tumors were scored as papillomas or carcinomas by morphological appearance on collection, followed by histological confirmation after H&E staining of paraffin sections. All animal experiments were performed in accordance with the United Kingdom Animal Scientific Procedures Act (1986).

**Generation of Murine Keratinocytes.** Primary murine keratinocytes were obtained from dorsal skin sections of 2-day-old mice essentially as described (16). Cells were plated in keratinocyte growth medium [modified MCDB 153 medium (Clonetics, San Diego, CA) containing the following supplements: 0.4% bovine pituitary extract, 10 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 50 ng/ml amphotericin-B, and 50 μg/ml gentamicin]. CaCl₂ solution was added to give a final Ca²⁺ concentration of 0.05 mm, and cells were maintained in a humid 37°C/5% CO₂ incubator. Primary cultures were trypsinized, and the trypsin was inactivated in medium containing 20% FCS before plating on fibronectin. For fibronectin plating experiments, dishes were coated by preincubating overnight at 4°C in a solution of 10 μg/ml fibronectin (Stratatec) in PBS before being used for cell plating.

**Protein Analysis.** For protein analysis, dishes of cells were washed in ice-cold PBS and either used immediately or flash frozen at −70°C. Cell monolayers were lysed in radioimmunoprecipitation assay buffer [50 mm Tris (pH 7.4), 150 mm NaCl, 5 mm EGTA, 0.1% SDS, 1% NP-40, and 1% sodium deoxycholate] with the inclusion of inhibitors (500 μm sodium fluoride, 1 mm phenylmethylsulfonyl fluoride, 100 μm sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml benzamidine, and 10 μg/ml aprotinin, Sigma Chemical Co.). Samples were sonicated and clarified by centrifugation at 21,000 × g at 4°C. Alternatively, frozen tumor samples were dissected into small particles before being suspended in radioimmunoprecipitation assay buffer and treated as above. Protein concentrations were measured by Micro bichinchoninic acid protein assay kit (Pierce), and samples were then suspended in sample buffer [50 mm Tris-Cl (pH 6.7), 2% SDS, 700 mm β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue] at 100°C for 2 min followed by analysis by SDS-PAGE. For Western blotting, proteins were transferred to nitrocellulose using a semidry blotting apparatus. Unoccupied binding sites were blocked with 5% dried milk powder in PBS + 0.1% Tween 20, followed by probing with either FAK mAb at 0.5 μg/ml (clone 77; Transduction Laboratories), anti-ERK (1:10,000; Sigma Chemical Co.), or antiphospho-ERK (Promega). Detection was by incubation with horseradish peroxidase-conjugated secondary antibody (New England Biolabs), and visualization was by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Results**

**Papilloma Formation Is Suppressed in fak +/− Mice.** Because circumstantial evidence links FAK protein expression with development of malignancy, we addressed whether variation in FAK expression levels in vivo would affect tumorigenesis. We first tested whether ablation of one copy of fak resulted in a similar reduction in FAK protein expression by comparing epidermal extracts from both fak +/+ and fak +/− mice using immunoblotting. Dorsal skin samples were collected, and epidermal proteins were prepared and pooled from four mice before Western blotting using an anti-FAK mAb as probe. As expected, and confirming previous reports (17), FAK protein expression was reduced in the epidermis of fak +/− mice, when compared with expression levels in the fak +/+ littermate controls (Fig. 1A, top panel). Comparison with another focal adhesion component that associates with FAK, namely c-Src, showed no detectable difference in expression between the fak +/− and fak +/+ mice (Fig. 1A, bottom panel).

fak +/− mice and wt littermates were treated with a single dose of DMBA followed by twice weekly application of TPA for 20 weeks. After the instigation of treatment, an early difference was observed in the benign tumor latency, with visible papillomas appearing in the fak +/+ mice at 5 weeks. In contrast, the fak +/− mice showed no visible tumor production until week 7 (Fig. 1B). In addition, 100% of mice in the control group had developed papillomas by week 11, whereas there was a delay of 5 weeks (until week 16) before 100% of the fak +/− mice also produced papillomas (Fig. 1B). More significantly, there was also a substantial and consistent reduction in the papilloma number per mouse in the fak +/− group, compared with the control fak +/+ group throughout the first 24 weeks of observation (Fig. 1C). The difference in papilloma numbers was evident as early as week 8, with the papilloma number per mouse at 18 weeks in the fak +/− mice showing a 46% reduction compared with the wt control group (P < 0.0001, by the Bonferroni two-sample t test; Fig. 1C). It is likely that this is a minimal estimate of suppression of papilloma formation because mice with high benign tumor loads were removed prematurely from the study.

**FAK Heterozygote Mice Show No Difference in Rate of Conversion to Malignant Carcinoma.** During chemical carcinogenesis, benign papillomas usually show an ~5% conversion rate to malignant squamous cell carcinomas (18). Mice were observed with regard to progression of papillomas to carcinomas on a weekly basis. Visual diagnosis was confirmed by histological examination of tumor sections (data not shown). Carcinomas were first observed on the fak +/+ mice at week 18; there was a delay period of 4 weeks until the first carcinoma was recorded in the fak +/− group at 22 weeks.
anti-ERK antibody to measure total ERK protein (Fig. 3, middle panel) to determine whether signaling to ERK was compromised by the reduced FAK protein expression in the fak +/+ keratinocytes (Fig. 3, bottom panel). Plating of keratinocytes from fak +/+ mice onto fibronectin caused a strong phosphorylation of ERK, clearly evident after 30 min and stronger after 60 min (Fig. 3, Lanes 3 and 4). In contrast, ERK phosphorylation and activation were suppressed and delayed in keratinocytes from fak +/−, with no enhanced phosphorylation evident after 30 min and only relatively weak phosphorylation evident after 60 min (Fig. 3, Lanes 1 and 2).

The attenuation of signaling to components of the pathway downstream of ras provides one possible explanation for the suppression of papilloma formation during chemically induced carcinogenesis, because activation of ras is a key step in tumor activation. This, together with the apparently elevated FAK protein expression in papillomas that do form, suggests that FAK protein expression must be maintained at a normal level to allow mutated H-ras to signal key changes within the cell that are required for tumor initiation.

Discussion

Much circumstantial evidence implicates FAK in the development of human malignancies. One theme linking many reports is the importance of raised FAK protein expression in cancer cells that correlates with invasive potential (22). Despite this, no experiments have been carried out to directly test how modulation of FAK protein in vivo affects tumor formation or progression. To address this, we have carried out chemical carcinogenesis experiments using mice that lack one copy of the fak gene. We have used skin carcinogenesis because it is well established in providing proof of the cause-effect relationship between carcinogen exposure, genetic alterations, and biological events leading to neoplasia (11).

We have used fak +/− mice to show that reduced FAK protein expression in the skin suppresses benign tumor formation (Fig. 1) but not malignant conversion of tumors that do form (Table 1). Specifically, there was a 46% reduction in papilloma formation after chemical treatment, when compared with the control group of mice that express wt levels of FAK (Fig. 1). Similar gene dosage effects, particularly of genes such as those encoding p21WAF and p53 that are implicated in cell growth control and survival, have been observed
previously in heterozygous mice, with p21WAF1/−/+ inducing an increase in papilloma formation, whereas the p53+/−/+ mice induced a decrease (13, 23).

A key event in the formation of DMBA-induced papillomas is the introduction of an activating mutation at codon 61 of the H-ras gene (13), implying that signaling downstream of ras was normal in keratinocytes derived from fak+/− heterozygous mice. We found that integrin-dependent stimulation of ERK/mitogen-activated protein-kinase was compromised in fak+/− keratinocytes when compared with fak+/+ wt keratinocytes after the replating of suspended cells on fibronectin (Fig. 3). This indicates that FAK protein expression may be limiting for integrin-dependent activation of the ras/ERK pathway, at least in keratinocytes. FAK is believed to be upstream of ras in signaling between integrins and the ras/ERK pathway, specifically via integrin-induced phosphorylation of FAK on tyrosine-925 providing a binding site for the SH2 domain of Grb-2, which subsequently recruits mSOS, leading to ras activation (21). Interestingly, it has been shown that activation of ERK by an activated mutant ras is still adhesion dependent (24), providing a possible explanation for the ability of reduced FAK protein expression to influence signaling in the presence of the DMBA-induced activating H-ras mutation present in papillomas and a rationale for the suppression of papilloma formation under these circumstances.

A possible role for elevated FAK protein expression in tumor cells is in the acquisition of the invasive phenotype. Cancer cell invasion is a complex process that requires cells to attach and migrate through underlying ECM and into surrounding tissue. Because FAK functions as a key regulator of ECM-dependent cell migration (5, 14, 25, 26) and because endogenous levels of FAK protein expression can limit the rate of cell motility in some cell types (25), it seems likely that elevated FAK protein in tumor cells may release normal constraints on the rate of cell motility and, in turn, facilitate invasion. However, our observation that the papilloma to carcinoma conversion frequency was similar in both the fak+/− heterozygous and fak+/+ wt mice groups seemed to indicate that FAK protein expression was not an important determinant of tumor progression. This interpretation was complicated by the finding that FAK protein expression in papillomas from both fak+/− and fak+/+ mice was indistinguishable (Fig. 2), indicating that FAK protein expression was elevated during papilloma formation despite only a single operational fak allele in fak+/− mice. This additionally suggests that there is a selection pressure for elevating FAK protein during tumor initiation, perhaps explaining why there is no constraint on additional progression to carcinoma in the fak+/− mice. How and why elevated FAK protein is selected for during papilloma formation remains unclear. One possibility is that raised FAK protein provides an advantage with regard to promoting cell growth or survival after induction of the activating H-ras mutation and that the low level of FAK protein in fak+/− heterozygous mouse skin is incompatible with tumor cell growth, or survival, during chemically induced transformation. This is compatible with FAK’s role in conferring the ability of transformed cells to grow in an anchorage-independent environment (2) and as a survival factor in adherent cells (27, 28).

In conclusion, the results we present here provide the first direct evidence for a cause and effect relationship between the level of FAK protein expression tumor formation in vivo. Whether there is also an in vivo role for FAK during malignant conversion remains to be tested by use of conditional fak knockout strategies.

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References


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