Focal Adhesion Kinase Overexpression Induces Enhanced Pathological Retinal Angiogenesis

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PURPOSE. Focal adhesion kinase (FAK) is involved in processes integral to angiogenesis, such as cell growth, survival, and migration. FAK is activated by angiogenic growth factors, such as insulin-like growth factor (IGF)-I, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). The first indication that FAK was involved in angiogenesis came from studies in the developing mouse embryo showing intense FAK immunoreactivity associated with the developing vasculature.10 Subsequent work showed that the FAK-deficient embryo could implant and initiate gastrulation normally, but showed numerous abnormalities, including poor vascularization.11 Experiments conducted with isolated endothelial cells support a role for FAK in endothelial cell survival,12 migration, and proliferation.13 Finally, angiogenic growth factors, such as platelet-activating factor (PAF),14 TNF-related activation-induced cytokine (TRANCE),15 vascular endothelial growth factor (VEGF),16,17 angiopoietin-1,18 and basic fibroblast growth factor (bFGF),19 are coupled to phosphorylation and activation of FAK. Insulin-like growth factor (IGF)-I, a potent angiogenic factor that promotes endothelial cell survival and enhances numerous phases of angiogenesis, either increases or decreases FAK phosphorylation, depending on the cell type and experimental conditions.8,20–22 Therefore, we sought to examine the role of FAK in key steps involved in angiogenesis. To accomplish this, we transiently transfected human retinal endothelial cells (HRECs) with FAK or FAK-related nonkinase (FRNK), an inhibitor of FAK,23–24 and examined the effect of FAK/FRNK overexpression on HREC migration and in vivo angiogenesis. Transfection of HRECs with FAK caused increased cell migration, whereas transfection with FRNK decreased cell migration. Finally, injection of a plasmid encoding FAK into mouse pup eyes led to a formation of large glomeruli-like vascular tufts and neovascularization in vivo, whereas injection of a plasmid expressing FRNK decreased neovascularization. This is the first demonstration that FAK/FRNK modulates retinal angiogenesis in vivo and may represent a future therapeutic target.

MATERIALS AND METHODS

Experimental Animals

All animal procedures used were in agreement with the NIH Guide for the Care and Use of Laboratory Animals, with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with institutional guidelines and approved by the University of Florida Institutional Animal Care and Use Committee. Timed-pregnant C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Cell Culture

Human eyes (four donors) were obtained from the National Disease Resource Interchange (Philadelphia, PA) within 36 hours of death. The procedure for obtaining and using donor eyes adhered to the guidelines of the Declaration of Helsinki for experiments involving human tissue. Human retinal endothelial cells were prepared and maintained as previously described.25 The identity of HRECs was validated by demonstrating endothelial cell incorporation of fluorescence-labeled, acetylated LDL and by fluorescence-activated cell-sorting analysis, as

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previously described. Cells in passages 3 to 5 were used in the studies.26

Plasmids
CDM8-human FAK was obtained from Steve Kanner (Bristol-Myers Squibb, Seattle, WA; GenBank accession number L13616; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). FRNK encompasses the final 359 amino acids of the carboxyl terminus of the FAK protein.23 The FRNK coding sequence was generated using PCR to amplify the FRNK portion of FAK located within CDM8-FAK. The primers were designed so there would be a BstXI site at the 5’ end and a NotI site at the 3’ end. The sense primer, which encompasses the ATG start codon located at position 2309, was 5’-CTCCTCGCGGCCCGCTCAGTGTGGTCTCGTCTG-3’. The antisense primer, which encompasses the stop codon located at position 3588, was 5’-CTCCTCCTTGATGGAGTCCAGAAGACAG-3’. The antisense primer, which encompasses the stop codon located at position 3588, was 5’-CTCCTCCTTGATGGAGTCCAGAAGACAG-3’. The antisense primer, which encompasses the stop codon located at position 3588, was 5’-CTCCTCCTTGATGGAGTCCAGAAGACAG-3’. The antisense primer, which encompasses the stop codon located at position 3588, was 5’-CTCCTCCTTGATGGAGTCCAGAAGACAG-3’.

Transient Transfection of HRECs
HRECs were grown to 70% confluence on 150-mm plates and transfected with CDM8, CDM8-FAK, or CDM8-FRNK (10 μg) using diethylaminoethyl (DEAE)-dextran as a carrier, as previously described.27-28 Cells were washed once with PBS and 10.5 mL of medium (10% Nusereum; BD Biosciences, Franklin Lakes, NJ) was added to the cells. A solution (324 μL total) of 10 μg of plasmid DNA in DEAE-dextran was added to the cells, followed by the immediate addition of 8.1 μL of 100 mM chloroquine (Sigma-Aldrich, St. Louis, MO). Cells were incubated for 4 hours at 37°C in 5% CO2. The plates were manually shaken every 15 to 30 minutes at 37°C. After 4 hours, the cells were shocked for 1 minute by the addition of 10% dimethyl sulfoxide (DMSO) in PBS, washed twice with PBS, and then placed in complete medium. Cells were harvested for the migration assays 48 hours after transfection.

Analysis of FAK/FRNK Expression by Western Blot Analysis
The cells were rinsed in phosphate-buffered saline (PBS) and lysed in ice-cold RIPA (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 5 mM EDTA, and 5 mM NaF) containing a 1:20 dilution of mammalian proteinase inhibitor cocktail (Sigma-Aldrich). Protein was assayed using the bicinchoninic assay (BCA) reagent (Pierce Biotechnology, Rockford, IL), and equal amounts of protein per lane (30 μg) were resolved by electrophoresis on a 10% polyacrylamide gel in denaturing and reducing conditions. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. FRNK (125 kDa) and FAK (125 kDa) were detected with a polyclonal antibody directed against human FAK (Upstate Biotechnology, Lake Placid, NY), goat anti-rabbit IgG, and the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ). This FAK antibody recognizes the C-terminal portion of FAK and thus reacts with FAK (125 kDa) and FRNK (44 kDa).

Effect of FAK/FRNK on Endothelial Cell Migration
This assay measures the ability of transfected HRECs to migrate through membranes in Boyden chambers in response to chemotactic agents. Cells were transfected with CDM8, CDM8-FAK, or CDM8-FRNK, as just described. After transfection, HRECs were trypsinized (trypsin-EDTA solution for endothelial cell culture; Sigma-Aldrich) until the cells became a single-cell suspension. Trypsin was then inactivated, and cells were washed three times in PBS and suspended in DMEM to a final concentration of 1000 cells/μL. Thirty thousand cells (30 μL) were added per lower well in the blind-well chemotaxis chamber. The wells were then overlaid with a porous polyvinyl- and pyrrolidone-free polycarbonate membrane (12-μm pores) coated with 10% bovine collagen. The chemotaxis chamber was inverted and incubated in a humidified atmosphere of 5% CO2 atmosphere air at 37°C for 4 hours to allow cells to attach to the membrane. Chambers were then placed upright, and 50 μL of a cocktail containing VEGF (25 ng/mL), bFGF (25 ng/mL), and various concentrations of IGF (1, 10, or 100 ng/mL) were added to the upper wells. The chambers were then incubated for 12 hours, as described earlier. Membranes were collected, and cells on the attachment (lower) side were scraped off leaving only those cells that migrated through the pores of the membrane onto the upper surface of the membrane. The cells on the membrane were then fixed in methanol, and stained (Leukostat solution; Fisher, Springfield, NJ) and then mounted on glass slides. DMEM was used as a negative control in each experiment to determine the amount of random cell migration, and DMEM with 10% FBS served as a positive control in each experiment. Each test condition was assayed with a minimum of six replicate wells. Cells were counted under a light microscope, and the number of migrating cells per well was calculated by averaging the number of cells counted in three separate high-power (400×) fields. The counts for the six replicate wells were then averaged, the statistical error calculated, and the results compared for statistical significance by Student’s t test.

Effect of FAK/FRNK Expression on Angiogenesis In Vivo
The neonatal murine model of hyperoxia-induced retinal neovascularization first described by Smith et al.25 was used to test the effects of local FAK and FRNK expression on angiogenesis. Newborn mice (postnatal day [P]10) were challenged with ice for 30 to 45 seconds to reduce their activity and then 0.5 μL of a mixture containing 1 μg CDM8, CDM8-FAK, or CDM8-FRNK and 0.1% (vol/vol) fluorescein sodium (Angiofluor; Alliance Pharmaceuticals, Richmond, TX) was injected into the vitreous of the right eye with a 52-gauge needle attached to a 10-μL syringe (Hamilton, Reno, NV). On P7, the animals were then placed in 75% O2 and maintained in this atmosphere until P12, at which time they were returned to normal air. At P17 the mice were anesthetized by subcutaneous injection with a mixture of ketamine-xylazine (10 mg/mL ketamine HCl, 2 mg/mL xylazine in 0.9% NaCl) at a dose of 5 μL/g body weight and then perfused via cardiac puncture with 3 mL of 4% formaldehyde in phosphate-buffered saline (pH 7.4; fixative) containing 50 mg/mL rhodamine-conjugated dextran (average molecular mass of 170 kDa; Sigma-Aldrich) or 50 mg/mL FITC-labeled dextran (average molecular weight of 1,000,000; Sigma-Aldrich). Eyes were then enucleated, pierced with a 30-gauge needle, and submerged in fixative for 30 minutes at room temperature. Selected eyes were washed in three changes of PBS over the course of 1 hour, after which the retinas were dissected and mounted flat in antifade medium (Vectorshield; Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy. Remaining eyes were embedded in paraffin for serial sectioning, as described by Smith et al.25 Sections were stained with hematoxylin and eosin to visualize cell nuclei. Individuals masked to the identity of the treatment counted all cell nuclei above the internal limiting membrane in 10 sections from each eye. Cross-sections that included the optic nerve were not sampled, because normal vessels emanated from the optic nerve. Although these normal vessels were distinguishable from neovascularization, they extended into the vitreous, fulfilling the counting criteria, and thus increasing the experimental error. Vascular cell nuclei were considered to be associated with new vessels if they were found on the vitreous side of the internal limiting membrane. Pericytes were not morphologically identifiable in the neovascular tufts. It is possible that pericytes or pericyte precursors were included in our cell counts.
FAK Immunohistochemistry

Sections of paraffin-embedded eyes were stained for FAK, as previously described.29 Sections were deparaffinized with xylene and hydrated through graded ethanol terminating in water. Antigen retrieval was then performed (Trilogy; Cell Marque, Hot Springs, AR) in a heated (90–95°C) water bath for 25 minutes. The sections were incubated in 0.1% Triton X-100 for 10 minutes, and endogenous peroxidases were then blocked by incubating the sections for 30 minutes in 3% hydrogen peroxide. The sections were blocked with normal serum for 1 hour. FAK was detected by using anti-FAK mouse monoclonal antibody clone 4.47 (Upstate Biotechnology) at a concentration of 5 μg/ml for 2 hours. This antibody reacts with human and mouse FAK and does not cross-react with FRNK, as it is directed against the N-terminal portion of FAK. The sections were washed in PBS, and staining was detected using the mouse the avidin-biotin complex (Vectastain Elite; Vector Laboratories). The chromogenic reaction was performed with 3,3’-diaminobenzidine. The sections were then counterstained with hematoxylin and dehydrated through graded ethanol terminating in xylene.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni correction, with either hyperoxia- or vehicle-treated results used as the determinant, as appropriate. Results are reported as the mean ± SE. P = 0.05 was deemed significant.

RESULTS

Effect of FAK/FRNK on Endothelial Cell Chemotaxis

This assay examined the ability of HRECs to migrate toward increasing amounts of IGF-I in the presence of a fixed amount of VEGF and bFGF. Under these conditions, IGF-I stimulated the migration of CDM8-transfected cells in a concentration-dependent manner. (Fig. 1A). Cells that were transfected with CDM8-FAK demonstrated a twofold increase in migration (102% ± 13%, P = 1.4 × 10⁻⁴) in response to 100 ng/mL IGF-I relative to cells transfected with CDM8. Conversely, CDM8-FRNK transfected cells demonstrated a reduction of migration (20% ± 8%, P = 0.01 at 100 ng/mL IGF-I) compared with control cells that were transfected with empty CDM8. Western blot analysis showed an increase in FAK and FRNK expression in transfected cells (Fig. 1B).

Effect of FAK/FRNK on In Vivo Retinal Neovascularization

The perfused retinas of the animals injected with CDM8 and exposed to hyperoxia showed increased fluorescence at the periphery and dilated vessels.29 The pattern of vascular development and neovascularization observed in the perfused retinas of eyes that were injected with CDM8-FAK revealed areas of increased neovascularization and vascular abnormalities reminiscent of vascular glomeruli (Figs. 2B–D). CDM8-FAK-injected eyes exhibited tortuosity of existing vessels and vascular abnormalities also suggestive of microaneurysms. In contrast, there was a decrease in the neovascular tufts in the CDM8-FRNK–injected mice (Fig. 3B). Confocal microscopy of the flatmounted retinas of CDM8-FAK–injected mice demonstrated that the vascular anomalies exhibited a single vessel leading into the vascular tuft and a single vessel exiting the tuft (Figs. 2E, 2F).

Quantitative assessment of the cell nuclei above the internal limiting membrane agreed with the qualitative assessment. Injection of CDM8-FAK resulted in an increased number of neovascular nuclei (57% ± 7%, P = 3 × 10⁻⁴) relative to animals injected with CDM8 (Fig. 4). The CDM8-FRNK-injected retinas of eyes that were injected with CDM8-FAK demonstrated a twofold increase in migration (102% ± 13%, P = 1.4 × 10⁻⁴) in response to 100 ng/mL IGF-I compared with control cells transfected with CDM8 (P = 0.01). Positive and negative control results were obtained in nontransfected cells. The negative control cells were assayed with DMEM only, and the positive control cells were assayed with DMEM plus 10% FBS. (B) Expression of FAK and FRNK in transfected HRECs. Transiently transfected HRECs were detached from their plates with trypsin and washed. A portion of these cells was used for the migration assay in (A) and the other portion was lysed in RIPA. Lysates containing equal amounts of protein were electrophoresed. The resolved proteins were transferred to PVDF membranes and FAK (125 kDa) and FRNK (44 kDa) were detected. Lanes contain protein isolated from HRECs transfected with CDM8-FAK, CDM8, and CDM8-FRNK. The identity of the middle set of bands, which are frequently observed with this antibody, is unknown.
jected eyes demonstrated a reduced neovascular response (55% ± 15%, $P = 5 \times 10^{-5}$; Fig. 4).

The typical retina of a noninjected animal exposed to hyperoxia demonstrated preretinal neovascularization (Fig. 5A). In contrast, the CDM8-FAK–injected animals demonstrated vascular glomeruli that were highly proliferative clusters of tortuous vessels appearing to occur in areas of the neural retina not typically observed in this model, such as in the inner nuclear layer (Fig. 5B). As shown in Figures 5C and 5D, vascular glomeruli also protruded through the inner limiting membrane. These abnormalities were not apparent in uninjected eyes (Fig. 5A). In addition, the retina of CDM8-FAK–injected animals demonstrated thickening in the inner limiting membrane, the inner plexiform layer, and the inner nuclear layer (Fig. 5B–5E).

FAK immunoreactivity was localized to the cytoplasm of the endothelial cells. Although this antibody is unable to differentiate endogenous mouse FAK from exogenous human FAK, there appeared to be more FAK immunoreactivity associated

**FIGURE 2.** Effect of FAK on in vivo retinal neovascularization. Rhodamine-labeled, dextran-perfused retinas of animals injected with CDM8 (A) and CDM8-FAK (B) revealed areas of increased neovascularization, vascular abnormalities resembling of vascular glomeruli, tortuosity of existing vessels, and vascular abnormalities suggestive of microaneurysms (B). Higher magnification of the flatmounted retinas of CDM8-FAK–injected mice demonstrated the vascular anomalies (C, D). Confocal microscopy of FITC-labeled, dextran perfused retinas demonstrated that the vascular anomalies exhibit a single vessel leading into the vascular tuft and a single vessel exiting the tuft (E, F). Magnification: (A, B) ×50; (C, D) ×100; (E, F) ×600.

**FIGURE 3.** Effect of FRNK on in vivo retinal neovascularization. Rhodamine-labeled, dextran-perfused retinas of animals injected with CDM8 (A) and CDM8-FRNK (B) shows decreased leakage in the CDM8-FRNK–injected retina. Magnification, ×50.
late angiogenesis in concert with soluble growth factors such as bFGF and VEGF.32

Overexpression of FRNK caused a 20% inhibition in cell migration in vitro, whereas it inhibited angiogenesis by 55% in vivo. These results are not surprising, as FAK is known to influence cell growth, migration, invasion, and survival in vitro.51 Therefore, FRNK-mediated inhibition of FAK activity in vivo would be expected to affect several aspects of angiogenesis.

The formation of new blood vessels is a complicated process. Angiogenesis involves endothelial cell proliferation, migration, and tube formation. These events require coordinated interactions between soluble mediators, the extracellular matrix, and endothelial cell surface receptors. Integrins, in part, mediate the adhesion of endothelial cells to the ECM. Evidence now suggests that endothelial αv integrins in particular regulate angiogenesis in concert with soluble growth factors such as bFGF and VEGF.52

FAK is a signaling molecule that provides a potential point of convergence between integrin-mediated signaling pathways and growth-factor–mediated signaling pathways. FAK is phosphorylated on Y397, which is the autophosphorylation and activation site of this molecule,29 in response to adhesion of HRECs to fibronectin-coated dishes (Kornberg LJ, Grant MB, unpublished observations, 2002). Adhesion-mediated phosphorylation and activation of FAK has been associated with migration and survival of numerous cell types, whereas detachment from the ECM leads to dephosphorylation of FAK and apoptosis of nontransformed cells.51

Cell migration involves cycles of attachment and detachment to contacts with the extracellular matrix. Several growth factors are known to stimulate the migration of endothelial cells. Angiopoietin (Ang)-1 is a strong inducer of endothelial cell sprouting, which is a first step in angiogenesis and neovascularization. Ang-1 induced directional and nondirectional migration of porcine endothelial cells with a concomitant tyrosine phosphorylation of FAK.16 Both phosphorylation and migration were dependent on PI3-kinase activity.16

VEGF is probably the best-known endothelial mitogen and motogen. VEGF can act through two cellular receptors: flk-1 (KDR) and flt-1. VEGF stimulates cell migration through the KDR receptor, and this interaction involves FAK.53–54 In addition, VEGF stimulates Src activity in chick embryo blood vessels and induces a physical association between FAK and integrin αvβ5.16 This supports the notion that FAK is a point of

FIGURE 4. Quantitative assessment the effect of FAK/FRNK on in vivo retinal neovascularization. Cell nuclei above the internal limiting membrane were counted and support the qualitative assessment of retinal flatmounts. Mice injected with CDM8-FAK (FAK; n = 16) demonstrated enhanced angiogenesis (P = 3 × 10^-5), whereas mice injected with CDM8-FRNK (FRNK; n = 10) showed a decreased number of neovascular nuclei compared with those injected with empty CDM8 (control; P = 5 × 10^-5).

with CDM8-FAK-injected eyes (Fig. 6A) than the noninjected eye from the same animal (Fig. 6B).

DISCUSSION

These results show that enhanced expression of FAK caused increased cell migration in response to growth factors (IGF-I, bFGF, and VEGF), whereas overexpression of FRNK, an inhibitor of FAK, reduced the ability of HRECs to respond to these growth factors. In addition, injection of a plasmid expressing FAK into a murine hypoxia-induced retinal neovascularization model enhanced preretinal and intraretinal angiogenesis and the formation of vascular abnormalities, whereas injection of a plasmid expressing FRNK significantly reduced angiogenesis.

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convergence between ECM receptors and growth factor receptors in endothelial cell signaling.

IGF-I is a potent growth and survival factor involved in numerous stages of angiogenesis. We have previously shown that IGF-I stimulates HREC migration and promotes survival by increasing phosphotidylinositol-3 kinase and Akt activity. IGF-I is necessary for induction of maximum neovascularization by VEGF, and this interaction is mediated in part by activation of p44/42 mitogen-activated protein kinase. Both IGF-I and insulin can increase or decrease FAK phosphorylation, depending on cell type and experimental conditions.

Although several angiogenic substances can regulate FAK phosphorylation and thus its activity, little is known about the regulation of FAK protein levels during angiogenesis or other physiologic processes. Studies in multiple laboratories have demonstrated that FAK mRNA and/or protein is increased in several human cancers. The biological factors contributing to the upregulation of FAK mRNA and protein in cancer are unknown. Conversely, endogenous FAK is a substrate for cellular proteases. FAK can be degraded during apoptosis, leading to the release of a FRNK-like peptide that is presumed to be biologically active. In the experimental systems described herein, the effect of ectopically expressed FAK/FRNK on endogenous FAK/FRNK is unknown. However, some of the mechanisms described may explain the apparent discrepancies in FAK/FRNK in Figure 1B (compare the FAK band in the FRNK-transfected cells with the FAK band in the CD8-transfected cells; compare the FRNK band in the FAK-transfected cells with the FRNK band in the CD8-transfected cells).

The first indication that FAK is involved in angiogenesis came from studies in developing mouse embryos, which showed intense FAK immunoreactivity, associated with the developing vasculature. The FAK knockout mouse was an embryonic lethal mutation due to a general mesodermal effect, which was reminiscent of the fibronectin knockout embryo. The FAK-/- embryo exhibited numerous abnormalities in the vasculature, including discontinuity in the dorsal aorta and lack of blood cells in the embryonic heart or vasculature. Fibroblasts derived from these embryos showed decreased migration. Finally, endothelial cells deficient in FAK expression or function displayed a severely reduced ability to form tubules in synthetic basement membrane (Matrigel; BD Biosciences) thus supporting the idea that the vascular effects in FAK-null mice result from the inability of FAK-deficient endothelial cells to organize themselves into vascular networks.

In this study, microinjection of plasmids expressing FAK into the neonatal murine hypoxia-induced retinal neovascularization model led to increased preretinal and intraretinal neovascularization and vascular abnormalities, including formation of glomeruli-like structures, vessel tortuosity, and formation of microaneurysms. Although these abnormalities have yet to be characterized entirely, they are reminiscent of the pathologic hypoxia-induced retinal angiogenesis that occurs in β integrin-null mice.

In the neonatal vascularization model, hypoxia is thought to induce the local release of growth factors such as VEGF, bFGF, and IGF-I, which act to induce angiogenesis and neovascularization in concert with ECM-derived signals. The ultimate amount of angiogenesis may depend on a limiting factor such as FAK. The addition of excess FAK to the system may lead to increased vascular abnormalities by creating an imbalance between growth factor and ECM-derived signals. Nonetheless, this is the first demonstration that FAK can stimulate retinal angiogenesis and neovascularization in vivo and suggests that inhibitors of FAK, such as FRNK, may have therapeutic potential in limiting the degree of retinal neovascularization.

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