Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK

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Abstract

Focal adhesion kinase (FAK) plays a critical role during vascular development because knockout of FAK in endothelial cells (ECs) is embryonic lethal. Surprisingly, tamoxifen-inducible conditional knockout of FAK in adult blood vessels (inducible EC–specific FAK knockout [i-EC-FAK-KO]) produces no vascular phenotype, and these animals are capable of developing a robust growth factor–induced angiogenic response. Although angiogenesis in wild-type mice is suppressed by pharmacological inhibition of FAK, i-EC-FAK-KO mice are refractory to this treatment, which suggests that adult i-EC-FAK-KO mice develop a compensatory mechanism to bypass the requirement for FAK. Indeed, expression of the FAK-related proline-rich tyrosine kinase 2 (Pyk2) is elevated and phosphorylated in i-EC-FAK-KO blood vessels. In cultured ECs, FAK knockout leads to increased Pyk2 expression and, surprisingly, FAK kinase inhibition leads to increased Pyk2 phosphorylation. Pyk2 can functionally compensate for the loss of FAK because knockdown or pharmacological inhibition of Pyk2 disrupts angiogenesis in i-EC-FAK-KO mice. These studies reveal the adaptive capacity of ECs to switch to Pyk2-dependent signaling after deletion or kinase inhibition of FAK.

Introduction

Vascular remodeling requires a complex interplay between growth factor receptors, extracellular matrix components, and integrin receptors, making these attractive targets for anti-angiogenic therapy. Key intermediary proteins function in a membrane-proximal manner to integrate extracellular signals and promote intracellular signal transduction required for vasculogenesis and angiogenesis. One of these intracellular proteins is the cytoplasmic tyrosine kinase FAK, which is activated by growth factor receptors or integrin clustering and is critical for the assembly of a variety of signaling complexes (Mitra and Schlaepfer, 2006). FAK expression is essential for blood vessel development because global (Ilic et al., 1995) or endothelial cell (EC)-specific (Shen et al., 2005; Braren et al., 2006) knockout of FAK results in embryonic lethality with vascular defects. Interestingly, overexpression of FAK has the opposite effect, as transgenic mice overexpressing FAK in ECs show enhanced angiogenic responses to skin wounds and muscle ischemia (Peng et al., 2004). Together, these studies point to FAK as a critical factor for developmental and pathological angiogenesis. Indeed, control of FAK signaling has been suggested as a potential anticancer therapy and several FAK inhibitors have recently been developed (Slack-Davis et al., 2007; Roberts et al., 2008). However, it is not clear whether FAK inhibitors target ECs or impact angiogenesis directly.

Because conditional knockout of FAK from the endothelium produces a lethal phenotype, the role of FAK during vascular remodeling in vivo has not been fully addressed. Here, we report that tamoxifen-inducible, Cre-mediated FAK deletion from adult endothelium is surprisingly not lethal due to functional compensation by the FAK-related protein proline-rich tyrosine kinase 2 (Pyk2). This compensatory switch from FAK to Pyk2 occurs in blood vessels and in cultured human ECs, promoting vascular hemostasis and preserving integrin-mediated signaling during vascular remodeling events.

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Abbreviations used in this paper: BAEC, bovine aortic endothelial cell; bFGF, basic fibroblast growth factor; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; i-EC-FAK-KO, inducible EC–specific FAK knockout; Pyk2, proline-rich tyrosine kinase 2; shRNA, short hairpin RNA; WT, wild type.

The online version of this paper contains supplemental material.
Robust angiogenic response in i-EC-FAK-KO mice

In contrast to previous EC-specific FAK knockout models with embryonic lethality (Shen et al., 2005; Braren et al., 2006), knockout of FAK in adult endothelium did not produce an overt phenotype in mice of either gender. This finding prompted us to challenge these mice with angiogenic growth factors to assess the role of FAK during angiogenesis. Matrigel containing bFGF or VEGF was injected subcutaneously to induce neovascularization. After 5 d, mice were injected with FITC-lectin to label ECs and the plugs were removed and homogenized to quantify the FITC-lectin content. Surprisingly, either bFGF or VEGF elicited a robust angiogenic response in i-EC-FAK-KO mice that was equivalent to or greater than that observed in WT mice (Fig. 1 A). Although neovascularization was evident by both EC-specific FITC-lectin binding and labeling with EC markers, vessels within i-EC-FAK-KO plugs did not stain positive for FAK (Fig. 1 B). This result

Results and discussion

Generation of mice with inducible, conditional FAK knockout

To assess the postdevelopmental role of FAK in adult blood vessels, we used a Cre/loxP strategy to create an inducible, conditional knockout of FAK in ECs. Floxed FAK mice containing two loxP sites flanking exon 3 of the FAK gene (Shen et al., 2005) were crossed with End-SCL-Cre-ER(T) mice containing tamoxifen-inducible Cre-ER(T) driven by the 5' endothelial enhancer of the stem cell leukemia locus (Gothert et al., 2004). At 5 wk of age, littermates of FAK fl/fl;Cre(+) and FAK fl/fl;Cre(−) mice were treated with 2 mg tamoxifen every 2 d for 2 wk to generate “wild-type” (WT) mice (tamoxifen-treated mice with no Cre expression and thus no FAK deletion) and inducible EC–specific FAK knockout (“i-EC-FAK-KO”) mice (tamoxifen-induced EC-specific Cre expression resulting in FAK deletion).
is expressed selectively on angiogenic EC in vivo (Brooks et al., 1994) and inhibitors of αvβ3 abolish angiogenesis in response to growth factors or tumors (Brooks et al., 1995), we examined the role of this integrin during angiogenesis in the presence or absence of FAK. In Matrigel plugs from WT and i-EC-FAK-KO mice, bFGF-stimulated blood vessels showed intense αvβ3 staining, indicating that FAK is not required for αvβ3 expression on angiogenic EC (Fig. 2 A). To assess the efficacy of αvβ3 antagonism, Matrigel plugs were implanted containing bFGF along with a cyclic RGD-fK peptide that selectively blocks αvβ3 function (Dai et al., 2000) or a control cyclic RAD-fK peptide. We found that the cRGD-fK peptide blocked angiogenesis in both genotypes (Fig. 2 B), which suggests that integrin-mediated signals can be transmitted through alternative intracellular components during angiogenesis in the absence of EC FAK.

Pyk2 is a cytoplasmic tyrosine kinase structurally related to FAK. FAK and Pyk2 have been proposed to promote both distinct and overlapping signaling events, which appear to vary by cell type. Although FAK is ubiquitously expressed, Pyk2 is selectively enriched in certain cell types, including hematopoietic cells (Tang et al., 2002). Although Pyk2 has recently been linked to EC functions including VE-cadherin–mediated adhesion (van Buul et al., 2005; Allingham et al., 2007), cell migration (Avraham et al., 2003), angiogenic sprouting (Tang et al., 2002; Matsui et al., 2007), and integrin-mediated signaling (Lamalice et al., 2007), the role of Pyk2 during vascular remodeling in vivo remains poorly understood. Although Pyk2 expression is enhanced in embryonic fibroblasts isolated from FAK null mice (Sieg et al., 1998), no changes in Pyk2 levels have been detected within tissues from conditional FAK knockout mice with the exception of conditional knockout in osteoblasts (Kim et al., 2007).

To determine if Pyk2 may account for the compensatory angiogenic response observed in adult i-EC-FAK-KO mice, tissues from WT or i-EC-FAK-KO mice were examined using...
The loss of FAK on blood vessels in vivo results in increased Pyk2 expression and pY402 phosphorylation (Fig. 3 D). Together, our results suggest that Pyk2 expression increases after the loss of FAK on blood vessels in vivo. This Pyk2 response appears to be an overcompensation resulting in robust angiogenesis and vascular leakage, possibly because of constitutively increased Pyk2 activity.

Pyk2 compensation for loss of FAK also occurs in human ECs

To investigate whether a similar compensatory mechanism might take place in human ECs, we knocked down FAK in primary human umbilical vein ECs (HUVECs) using short hairpin RNA (shRNA) and monitored expression of Pyk2 over time. Compared with a nonsilencing scramble control shRNA (Scr), FAK shRNA
induced an 85–90% knockdown of FAK along with a threefold induction of Pyk2 expression at 7 d (Fig. 3 E). These findings are consistent with the increase in Pyk2 we observed in i-EC-FAK-KO mice, a model in which Cre-mediated FAK deletion occurs gradually during a 2-wk tamoxifen treatment period. The fact that compensatory Pyk2 expression increases only after 3–7 d may explain the lethality associated with EC FAK deletion during development (Shen et al., 2005; Braren et al., 2006), a situation in which the loss FAK cannot be compensated for by Pyk2 on a timescale to allow embryonic survival.

**FAK inhibition activates Pyk2**

Typical ECs express low levels of Pyk2, as observed for the mouse heart (Fig. 3, A–C) and HUVECs (Fig. 3 E). In contrast, bovine aortic ECs (BAECs) express significant levels of endogenous Pyk2 (Fig. 3 F). Treatment of these Pyk2-positive ECs with the NVP-TAC544 FAK inhibitor induced a dose-dependent decrease in FAK pY397 phosphorylation along with a surprising increase in Pyk2 pY402 phosphorylation (Fig. 3 F). In vitro kinase assays confirm that this inhibitor blocks FAK while increasing Pyk2 in BAECs (Fig. S1 C). Although it is possible that this inhibitor may interact with Pyk2, leading to its activation, we observed similar Pyk2 activation in mouse embryonic fibroblasts treated with two chemically distinct FAK inhibitors (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1). Together, our in vitro and in vivo studies using genetic and pharmacological approaches suggest that Pyk2 activity increases upon FAK deletion or inhibition and that a balance of FAK/Pyk2 activity may influence EC growth or survival during angiogenesis.

**Pyk2 can functionally compensate for loss of FAK during angiogenesis**

To further manipulate FAK and Pyk2 function during angiogenesis, we grew sections of mouse aorta in a 3D Matrigel culture to examine EC sprouting over time. Consistent with our in vivo results (Fig. 1), we observed equivalent ex vivo sprouting between genotypes, and immunohistochemical staining confirmed the absence of FAK on vessels sprouting from rings isolated from i-EC-FAK-KO mice. Bars: (A) 0.5 mm; (C) 80 μm.
selective FAK deletion within ECs. The shRNA-mediated FAK knockdown results are consistent with our observations for sprouting in vivo (Fig. 1E), in which pharmacological FAK inhibition delivered to all cell types did not negatively affect the angiogenic response in i-EC-FAK-KO mice. Together, these studies strongly suggest that EC FAK expression is responsible for the antiangiogenic properties of FAK inhibitors in WT mice, whereas Pyk2 expression is responsible for angiogenesis in i-EC-FAK-KO mice.

To further test this, aortic explants were treated daily with an ATP-competitive inhibitor selective for FAK but not Pyk2 (PF-228; Slack-Davis et al., 2007) or a dual inhibitor of FAK/Pyk2 (PF-271; Roberts et al., 2008). The effects of these inhibitors on FAK and Pyk2 tyrosine phosphorylation are shown in Fig. S2. In aortic vessels isolated from WT mice, both inhibitors reduced the amount of angiogenic sprouting to a similar extent (Fig. 5C). However, only the dual FAK/Pyk2 inhibitor could block sprouting in i-EC-FAK-KO explants. n = 6 each; *, P < 0.05 versus control shRNA or vehicle. Graphs show mean ± SEM. (D) Our results suggest a necessity for FAK and/or Pyk2 expression in ECs that can preserve the angiogenic response in i-EC-FAK-KO mice.

Figure 5. Pyk2 can functionally compensate for loss of FAK during angiogenesis. (A and B) Knockdown of FAK (but not Pyk2) slowed the rate of sprouting in WT aortic explants, indicating FAK plays the primary role during angiogenesis in normal blood vessels. For i-EC-FAK-KO, knockdown of Pyk2 (but not FAK) inhibited the angiogenic response. Broken lines indicate sprout length. Bar, 0.25 mm. (C) Sprouting of WT aortic explants was dose-dependently blocked by either a pharmacological inhibitor selective for FAK (PF-228) or a dual FAK/Pyk2 inhibitor (PF-271). Only the dual FAK/Pyk2 inhibitor (PF-271) blocked sprouting in i-EC-FAK-KO explants. n = 6 each; *, P < 0.05 versus control shRNA or vehicle. Graphs show mean ± SEM. (D) Our results suggest a necessity for FAK and/or Pyk2 expression in ECs that can preserve the angiogenic response in i-EC-FAK-KO mice.
reduce sprouting in aortic explants isolated from i-EC-FAK-KO mice (Fig. 5 C). Thus, we have demonstrated using both genetic and pharmacological approaches that increased Pyk2 expression and kinase activity compensate for the loss of FAK within i-EC-FAK-KO mice during angiogenesis. These findings reveal that Pyk2, typically involved in hematopoietic signaling, can adapt to assume the function of FAK within ECs to preserve the angiogenic response in vivo.

Conclusions

We have demonstrated that although normal ECs express low levels of Pyk2, increased Pyk2 expression and activity occurs upon loss of FAK both in i-EC-FAK-KO mice and in cultured ECs treated with FAK shRNA (summarized in Fig. 5 D). In cells with endogenous Pyk2, Pyk2 activity dose-dependently increases upon a 1-h treatment with pharmacological FAK inhibitors (Figs. 3 F, S1 C, and S2). Our findings support the hypothesis that ECs have the capacity to use Pyk2 signaling, but Pyk2 may be suppressed by FAK under normal conditions. Accordingly, the mechanism by which Pyk2 is up-regulated after FAK blockade warrants further investigation. We recently reported that Pyk2 expression is elevated in primary mouse and human fibroblasts upon FAK silencing and linked to enhanced p190RhoGEF expression leading to deregulated RhoA activation, elevated focal adhesion formation, and enhanced cell proliferation (Lim et al., 2008). Future work will reveal whether fibroblasts, ECs, or even carcinoma cells may share similar pathways of FAK/Pyk2 regulation and how these systems may contribute to the angiogenic response during cancer. This is especially relevant to the further development of dual FAK/Pyk2 inhibitors as anticancer or, potentially, antiangiogenic drugs.

Materials and methods

Mice

All mice were maintained on a C57Bl6 background. Floxed FAK mice (Shen et al., 2004) were crossed with mice harboring tamoxifen-inducible CreER(T) driven by the S£ exon enhancer of the Stem cell leukemia locus (provided by C.G. Begley, University of Western Australia, Perth, Australia; Gothert et al., 2004). Age-matched littermates of FAK fl/fl/Cre/+ and FAK fl/fl/Cre– mice were treated with 2 mg tamoxifen (Sigma-Aldrich) every 2 d for 2 wk to generate WT mice (tamoxifen-treated mice with no Cre expression) and iEC-FAK-KO mice (tamoxifen-induced EC-specific Cre expression and FAK deletion).

Inhibitors and shRNA

The cyclic RGD-K peptide that binds integrin b3 with high potency and selectivity was synthesized as described previously (Dai et al., 2000). The FAK inhibitor NVP-TAC544 (Garaco-Echevarria et al., 2004, 2005) provided by T. Honda (Novartis, Tsukuba, Ibaraki, Japan) was solubilized in Cremophore EL/DMSO/ethanol (1:1:1) and injected i.p. with 40 mg/kg daily (850 nM). The dual FAK/Pyk2 inhibitor PF-271 (Roberts et al., 2008) was synthesized according to available methods (Kath and Luzzio, 2004; Kath et al., 2005). The FAK inhibitor PF-228 (Slack-Davis et al., 2007) was provided by W.G. Roberts (Pfizer, Groton, CT). Both inhibitors were solubilized in DMSO at 1 mM. Lentiviral expression of scramble control, FAK, and Pyk2 shRNA has been described previously (Schloepfer et al., 2007).

Primary antibodies

Antibodies were obtained from Santa Cruz Biotechnology, Inc. (FAK, paxillin, and Erk2), Cell Signaling Technology (Pyk2, p130Cas pY249/pY140, and Src pY416), BD Biosciences (Pyk2 and integrin β3), Millipore (FAK, Pyk2 pY402, and paxillin pY118)), Invitrogen (FAK pY397 and Pyk2 pY402), and Sigma-Aldrich (actin). Blood vessels were labeled for “EC markers” with a mix of rat anti–mouse antibodies recognizing Flk-1 (BD Biosciences), CD31 (BD Biosciences), VE-cadherin (BD Biosciences), and CD105 (Millipore).

In vivo angiogenesis

The Matrigel assay was performed to assess in vivo angiogenesis (Weis, 2007). In brief, mice were injected subcutaneously on the flank with 400 µl growth factor–reduced Matrigel (BD Biosciences) containing either sterile saline or 400 ng of human recombinant bFGF (Millipore) or VEGF (PeproTech). After 7 d, mice were injected intravenously with 20 µg FITC-conjugated lectin that binds selectively to mouse ECs (GSL I–BSL I; Vector Laboratories). The Matrigel plugs were removed, photographed, viewed whole-mount, and then fixed and stained for microscopy. Alternatively, plugs were homogenized and the fluorescence content was read at 620 nm (excit) or hemoglobin content was quantified using the QuantChrom hemoglobin assay kit (BioAssay Systems).

In vivo permeability

A modified Miles assay was used to evaluate VEGF-induced leak in the skin as described previously (Elce et al., 1999).

Ex vivo angiogenesis

The abdominal aorta was isolated using sterile technique and cut into 1-mm sections that were embedded in growth factor–reduced Matrigel and cultured using DME with 10% FCS and 30 ng/ml human recombinant VEGFA-165 (PeproTech) daily. Images were acquired on an inverted microscope (Axiovier 100; Carl Zeiss, Inc.) using a 20× 0.70 NA objective (Carl Zeiss, Inc.) and a SPOT RT camera (Model 2.2.1; Diagnostic Instruments, Inc.). Image J (National Institutes of Health) was used to measure sprout length.

Immunoblotting and immunostaining

Standard Western blotting and immunostaining of cells, whole-mount preparations, or frozen sections was performed. Secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen) were used for immunofluorescence. Images were acquired at room temperature using confocal microscopy (Nikon C1si with EZC1 acquisition software; Nikon) with Plan Apo 10x 0.45 NA air, Plan Apo 20x 0.75 NA air, and Plan Apo 60x 1.40 NA oil objective lenses (Nikon). Colocalization with EC markers was measured using MetaMorph 7 (MDS Analytical Technologies).

Statistical analysis

Graphs are presented as mean ± SEM, with statistical significance determined from a two-tailed Student’s t test using α = 0.05 and P < 0.05.

Online supplemental material

Fig. S1 provides the chemical structure and kinase profile for the NVP-TAC544 FAK inhibitor. Fig. S2 reveals the dose response of FAK inhibitors on FAK and Pyk2 tyrosine phosphorylation. Fig. S3 offers validation of the Pyk2 shRNA. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200710038/DC1.

We give thanks to Drs. M. Hogan and B. Majeti for technical assistance and Dr. C. Glenn Begley for End-SCL-Cre mice.

S.-T. Lim received a postdoctoral fellowship from the American Heart Association (grant AHA0725169Y). D.D. Schlaepfer is an Established Investigator of the American Heart Association (grant AHA05540115N). D.A. Cheresh was supported by National Institutes of Health (NIH) grants CA50286, CA45726, and HL78912, and D.D. Schlaepfer was supported by NIH grant CA102310.

Submitted: 5 October 2007
Accepted: 7 March 2008

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