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Integrin-Linked Kinase in the Vascular Smooth Muscle Cell Response to Injury

Bernard Ho,* Guangpei Hou,*
J. Geoffrey Pickering,[†] Gregory Hannigan,[‡]
B. Lowell Langille,^{*§} and Michelle P. Bendeck*

From the Department of Laboratory Medicine and Pathobiology,* University of Toronto, Toronto, Ontario, Canada; the Robarts Research Institute, and the Department of Medicine,[†] University of Western Ontario, London, Ontario, Canada; Monash Institute of Medical Research, Monash University, Melbourne, Australia;[‡] and Toronto General Research Institute,[§] University Health Network, Toronto, Ontario, Canada

Integrin-mediated interactions between smooth muscle cells (SMCs) and the extracellular matrix regulate cell migration and proliferation during neointimal hyperplasia. Integrin-linked kinase (ILK) is a serine-threonine kinase and scaffolding molecule that acts downstream of integrin receptors to modulate cell adhesion; therefore, we examined ILK function in SMCs during wound repair. Silencing of ILK expression with siRNA *in vitro* decreased cell adhesion to fibronectin and accelerated both cell proliferation and wound closure in the cell monolayer; it also resulted in the rearrangement of focal adhesions and diminished central actin stress fibers. Akt and GSK3 β are ILK substrates that are important in cell motility; however, ILK siRNA silencing did not attenuate injury-induced increases in Akt and GSK3 β phosphorylation. Following balloon catheter injury of the rat carotid artery *in vivo*, a dramatic decrease in ILK levels coincided with both the proliferation and migration of SMCs, which leads to the formation of a thickened neointima. Immunostaining revealed decreased ILK levels in the media and deep layers of the neointima, but increased ILK levels in the subluminal layers of the intima. Taken together, these results suggest that ILK functions to maintain SMC quiescence in the normal artery. A decrease in ILK levels after injury may permit SMC migration, proliferation, and neointimal thickening, and its re-expression at the luminal surface may attenuate this process during later stages of the injury response. (Am J Pathol 2008, 173:278–288; DOI: 10.2353/ajpath.2008.071046)

Vascular smooth muscle cell (SMC) proliferation, migration, and extracellular matrix synthesis contribute to arterial wound repair and the development of neointimal hyperplasia in important vascular pathologies, including restenosis and atherosclerosis. Interactions between SMCs and the extracellular matrix regulate these processes through binding of the integrin family of cell adhesion receptors. A key mediator of integrin signaling is integrin-linked kinase (ILK), a serine-threonine kinase that interacts with the cytoplasmic domain of β_1 - and β_3 -integrin subunits.¹ ILK is also an important scaffolding protein at focal adhesions, where it links integrins to the actin cytoskeleton and regulates actin polymerization.² In many cell types, ILK is activated by phosphatidylinositol 3-kinase (PI3K), and regulates the activity of the downstream kinases protein kinase B/Akt (Akt) and glycogen synthase kinase 3 β (GSK3 β) through phosphorylation.^{3,4} ILK has been implicated in processes involving cell proliferation and motility, including anchorage-independent growth, survival, and cell cycle progression,^{5,6} as well as the epithelial-mesenchymal transition, invasion, and migration.⁷ Evidence from genetic mouse models demonstrates important roles for ILK in vascular development,^{8,9} tumor angiogenesis,^{10,11} and cardiac hypertrophy.^{12–14} In SMCs, ILK phosphorylates myosin light chain and myosin phosphatase and may therefore influence cytoskeletal function and cell contraction.^{15–17} Despite this research, little is known about ILK in arterial wound repair and intimal hyperplasia.

In this study, we used knockdown strategies to examine ILK function following SMC injury and wound repair *in vitro*. ILK expression and activity were measured follow-

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Address reprint requests to Michelle P. Bendeck, Ph.D., Professor, Career Investigator, Heart and Stroke Foundation of Ontario, Department of Laboratory Medicine and Pathobiology, University of Toronto, Medical Sciences Building, Rm. 6213, 1 King's College Circle, Toronto, ON, Canada M5S 1A8. E-mail: michelle.bendeck@utoronto.ca.

ing balloon catheter injury of the rat carotid artery, a model used to study SMC responses to injury.

Materials and Methods

Antibodies

Polyclonal antibodies from Cell Signaling Technology included anti-ILK antibody (#3862), anti-phospho-Akt (Ser473) (#9271), anti-Akt (#9272), and anti-phospho-GSK3 β (Ser9) (#9336). The monoclonal anti-GSK3 β antibody (clone 4G-1E), and the polyclonal anti-ILK antibody (#06-592) used for immunoprecipitation were from Upstate Cell Signaling Solutions. Rabbit polyclonal anti- β -actin antibody (ab8227) was from Abcam, and the mouse monoclonal anti- α -actin antibody (clone asm-1) was from Boehringer Mannheim Biochemica.

SMC Wound Assay, Proliferation Assay, and ILK siRNA Treatment

To examine the role of ILK in the SMC response to injury, we performed a scrape wound assay on confluent SMCs grown *in vitro*, an assay that mimics the cell migration and proliferation that occurs during intimal hyperplasia. Primary medial SMCs were obtained from the rat carotid artery, and propagated in culture as previously described.¹⁸ Cells were used from passages 4 to 10. Cells were grown to confluence in a 6-well tissue culture plate, then wounded with a P20 pipette tip to create a lengthwise wound measuring ~300 μ m in width, and incubated in standard growth media, Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2% penicillin-streptomycin. Measurements of wound width were obtained every 12 hours over a period of 60 hours using Simple PCI Imaging software (C-Imaging, Mars, PA). Mean values were expressed as a percent wound closure, which was calculated as follows: Percent wound closure = $1 - (\text{width}_t / \text{width}_0) \times 100\%$. These experiments were performed in duplicate and repeated three times.

To determine the importance of ILK in regulating wound closure, ILK expression in SMCs was attenuated by RNA interference. SMCs were transfected in suspension with either a non-targeting control siRNA (Dharmacon siCONTROL) as a control for non-sequence-specific effects, or an ILK-specific siRNA sequence (Silencer Pre-designed siRNA ID #56983 from Ambion, Austin TX). The ILK siRNA targets sequence in exon 8 of the rat mRNA for ILK (NM_133409). For each well, 90 pmol of either the non-targeting or ILK-specific duplex was diluted in 500 μ l Opti-MEM I reduced serum medium and 5 μ l of Lipofectamine RNAiMAX was added. The mixture was gently mixed and allowed to incubate for 20 minutes at room temperature. SMCs grown in T75 tissue culture flasks were trypsinized, spun down, and resuspended in standard growth medium without antibiotics such that 2.5 ml media contained 200,000 cells. To each well containing RNAi duplex-Lipofectamine RNAiMAX complexes, 200,000 cells were added, to give a final RNA concen-

tration of 30 nmol/L. Twenty-four hours after transfection, antibiotic-free medium was replaced with standard growth medium. Once the cells reached confluence, they were wounded. Attenuation of ILK expression was verified by Western blotting of cell lysates probed with antibody against ILK.

To measure cell proliferation, SMCs were transfected and plated in 6-well plates as described above. Twenty-four hours after transfection, the antibiotic-free medium was replaced with media containing antibiotics and 0.5% serum to starve the cells for 16 hours. The cells were then wounded using a comb with multiple teeth each 1 mm wide, and media was replaced with standard growth medium containing 20 μ l/ml Alamar Blue (Biosource, Camarillo, CA 93012) and incubated for 1 hour. Media was removed and the fluorescence was read at excitation 560 nm and emission 590 nm using a Fluostar Optima fluorescence reader (BMG Lab Technologies). Cells were replenished with fresh media, and the Alamar Blue procedure was repeated at 24 and 48 hours after injury. Blank values read from wells containing no cells were obtained at each time point and were subtracted, and the ratio of the fluorescence measured at 24 or 48 hours relative to the measurement at 1 hour was calculated. Wells were assayed in triplicate, and the entire experiment was repeated three times.

SMC Attachment Assay

Attachment assays were performed in 96-well plates pre-coated with 10 nmol/L fibronectin as previously described.¹⁹ SMCs were treated with either non-targeting or ILK siRNA as described above, then 60,000 cells were added to each well, and the cells were allowed to attach for 90 minutes. Nonadherent cells were washed off with PBS, the remaining cells were stained with 0.5% toluidine blue, and the absorbance of the solution in the well was measured at 595 nm in a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The experiment was done in duplicate and repeated three times.

Fibronectin Assembly Assay

Following incubation with either control non-targeting siRNA or ILK siRNA, subconfluent layers of SMCs were plated on 35-mm dishes. Oregon-green-labeled human plasma fibronectin (provided by G. Pickering²⁰) was added to the cells at a concentration of 100 nmol/L in standard growth medium, and the cells were incubated for 24 hours. The cells were washed with PBS to remove non-assembled fibronectin, then fixed with 4% paraformaldehyde for 15 minutes, and stained with Hoechst 33258 for 30 minutes to label cell nuclei. This experiment was repeated twice.

Subcellular Localization of ILK, Paxillin, and Actin

Subconfluent cultures of rat SMCs were stained with antibodies against ILK and paxillin to determine the local-

ization of ILK and focal adhesion complexes. Cells were transfected with control siRNA or ILK siRNA as described above, then plated on glass coverslips in 24-well plates; 24 hours after transfection the antibiotic-free medium was replaced with standard growth medium, and the cells were incubated for an additional 18 hours. Cells were washed with PBS then fixed with methanol and 4% paraformaldehyde at a 1:1 dilution for 15 minutes. The cells were stained with anti-ILK and anti-paxillin (BD Bioscience #650051) antibodies at dilutions of 1:100 for 16 hours at 4°C. Cells were then incubated with secondary antibodies [CY3-conjugated anti-rabbit antibody and fluorescein isothiocyanate-conjugated anti-mouse antibody, both at 1:200 in PBS], and with Hoescht nuclear stain (Hoescht 33258, Sigma, Saint Louis, MO) diluted 1:1000 for 30 minutes. Cells were also double stained with ILK antibody and tetramethylrhodamine isothiocyanate-phalloidin to stain actin filaments. For these experiments, cells were fixed in 4% paraformaldehyde and methanol at a ratio of 2:1 for 15 minutes. The ILK antibody was applied as described above, and tetramethylrhodamine isothiocyanate-phalloidin (Sigma #P1951) was applied at a 1:500 dilution. The secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch #111-095-045). Cells were visualized using an Olympus Fluoview confocal microscope.

Grid Wounding of Vascular SMC Cultures to Measure Akt and GSK-3 β Phosphorylation

SMCs were grown to confluence in 60-mm tissue culture dishes, then wounded along perpendicular axes using a sterilized comb (37-mm wide with 13 $\times$ 1-mm teeth) to create a grid wound. After wounding, fresh media was added and SMCs were allowed to proliferate and migrate for periods of 5 minutes, 30 minutes, and 1, 2, 4, 8, and 24 hours. Cells were lysed in 500 μ l lysis buffer, and the lysates were used for Western blotting to measure phospho-Akt, Akt, phospho-GSK3 β , GSK3 β , and β -actin.

siRNA was also used to determine whether ILK mediated the phosphorylation of Akt and GSK3 β in the wounded SMC cultures. In 60-mm culture dishes, 180 pmol of either the non-targeting or ILK-specific duplex was diluted in 1 ml Opti-MEM I reduced serum medium. Ten μ l of Lipofectamine RNAiMAX was then added to each dish. The mixture was gently mixed and allowed to incubate for 20 minutes at room temperature. SMCs grown in T75 tissue culture flasks were trypsinized, spun down, and resuspended in standard growth medium without antibiotics, such that 5 ml media contained 400,000 cells. To each dish containing RNAi duplex-Lipofectamine RNAiMAX complexes, 400,000 cells were added yielding a final RNA concentration of 30 nmol/L. The antibiotic-free medium was replaced with standard growth medium, 24 hours after transfection. Cells were grown to confluence and were then subject to grid wounding, 72 hours after transfection. Cells were lysed at various time points after injury, and the lysates were

subject to Western blotting and probed with antibodies against phospho-Akt, phospho-GSK3 β , and β -actin.

Western Blotting

Western blots containing cell lysates (10 μ g total protein per lane) were incubated for 16 hours at 4°C with anti-ILK, anti-phospho-Akt, anti-Akt, anti-phospho-GSK3 β , or anti-GSK3 antibodies diluted 1:1000 in blocking buffer. Secondary antibodies used were horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies at a dilution of 1:2000. Detection was performed using enhanced chemiluminescence (PerkinElmer Inc., Boston, MA). Measurements of band density were performed using Image J for Windows software (National Institutes of Health). ILK, phospho-Akt and phospho-GSK3 β were normalized to β -actin, total Akt and GSK3 β , respectively, and then further normalized to a single sample loaded on all immunoblots. Each experiment was repeated three to four times.

Arterial Injury

Male Sprague-Dawley rats (Charles River, Constant, QB), 3 to 4 months old and weighing 350 to 400g were used. Animal experiments were performed in accordance with the guidelines of the Canada Council on Animal Care. Rats were anesthetized by intraperitoneal injection of xylazine (4.6 mg/kg body weight, Rompum; Bayer Inc., Etobicoke, ON) and ketamine (70 mg/kg body weight, Ketaset; Ayerst Veterinarian Laboratories, Guelph, ON). Balloon catheter injury of the left carotid artery was performed as previously described.²¹ Rats were sacrificed by an overdose of ketamine (350 mg/kg), the common carotid arteries were cleared *in situ*, and the central portion 20 mm in length was removed and snap-frozen in liquid nitrogen. Each artery was pulverized in liquid nitrogen and the powder was collected in 100 μ l lysis buffer, composed of 34.7 M SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 23.4 μ mol/L leupeptin in 44.5 mmol/L Tris pH 7.6. Samples were centrifuged at 15,000 relative centrifugal force for 5 minutes at room temperature. Total protein content in the supernatant was measured using the BioRad Detergent-Compatible Microplate Assay. The remainder of the supernatant was diluted 1:1 with 2 $\times$ sample buffer composed of 0.1 M Tris pH 6.8, 69.4 mmol/L SDS, 20% glycerol and 0.1% bromophenol blue. Native SDS-polyacrylamide gels were loaded with 10 μ g of protein per lane, and subject to electrophoresis followed by transfer of proteins from the gel to polyvinylidene difluoride membranes. The western blots were incubated for 16 hours at 4°C with anti-ILK antibodies diluted 1:1000 in blocking buffer. Secondary antibodies were conjugated with horseradish peroxidase, and detection was performed using enhanced chemiluminescence exposing autoradiographs. Blots were stripped and reprobed with antibody against Akt to ensure even loading of proteins in the lanes, and were finally stained with Ponceau to confirm even protein loading. Measurements of band density on the autoradiographs were

made using Image J for Windows. All values were expressed relative the uninjured control carotid value on the same blot. These analyses included at least four rats per time point; thus the values presented represent the mean $\pm$ SEM from four animals.

Intimal Harvest

Fourteen days after balloon catheter injury, arteries were excised and cleared. The arteries were cut open longitudinally and pinned lumen side up onto silicone-coated plates. The intima was harvested by scoring the vessel at one end, then grasping the edge of the intima with forceps placed across the width of the vessel and peeling back the intima. The intima and media of each artery was lysed separately in 50 μ l lysis buffer. Protein was quantified as described above, and ILK expression in intimal and medial lysates was assessed by western blotting. These analyses included at least three rats.

ILK Kinase Assay

An assay to measure ILK activity was performed using myelin basic protein (MBP) as a substrate for phosphorylation by ILK. Uninjured rat common carotid arteries, and arteries harvested 14 days after balloon catheter injury were lysed in 100 μ l Nonidet P-40 (NP-40) lysis buffer composed of 145 mmol/L sodium chloride, 1% NP-40, 11.7 mmol/L sodium deoxycholate, 48.4 mmol/L Hepes pH 7.5, 2.34 μ mol/L leupeptin, 154 nmol/L aprotinin, and 3 mmol/L phenylmethylsulfonyl fluoride. ILK was immunoprecipitated from 160 μ g of total protein from arterial lysates by incubating with 1.44 μ g rabbit polyclonal anti-ILK antibody (Upstate Cell Signaling) at 4°C for 16 hours. Immune complexes were collected in 50 μ l of 50% protein A agarose in NP40 lysis buffer, rotated at 4°C for 2 hours, then washed with NP40 lysis buffer, and then kinase wash buffer (10 mmol/L MgCl₂, 10 mmol/L MnCl₂, 50 mmol/L Hepes pH 7.5, 0.1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol). Twenty-five μ l radioactive reaction buffer (10 mmol/L MgCl₂, 10 mmol/L MnCl₂, 50 mmol/L Hepes pH 7.5, 1 mmol/L sodium orthovanadate, 2 mmol/L sodium fluoride) was added to the reaction and combined with 5 μ g MBP and 5 μ Ci [γ -³²P] ATP. After a 30-minute incubation at 37°C, the reaction was terminated with 8 μ l of 5 $\times$ SDS sample buffer; 35 μ l of reaction volume was loaded into each well and separated on 12% SDS-polyacrylamide electrophoresis gels. The gels were dried and phosphorylation of MBP was detected by autoradiography. ILK kinase activity was normalized to total ILK protein expression as assessed on a Western blot prepared using total arterial lysates of the same arteries. Measurements of band density in autoradiographs and Western blots were performed using Image J for Windows software. The band density in autoradiographs was divided by the band density of the corresponding sample in Western blots, yielding a measure of ILK kinase activity per unit of ILK protein. Experiments included at least four rats.

Immunohistochemistry on Arterial Cross-Sections

Rats were subject to balloon catheter injury of the rat carotid artery then sacrificed at 2, 7, or 14 days after injury. Immediately after sacrifice of the rat the carotid arteries were perfusion-fixed with 4% paraformaldehyde at constant physiological pressure (110 mmHg) for 10 minutes. Arteries were embedded in paraffin and cut into 5 μ m-thick cross-sections. Sections were deparaffinized in xylenes, rehydrated in a gradient of anhydrous ethanol and rinsed with PBS. For antigen retrieval, sections were immersed in 10 mmol/L sodium citrate (pH 6.0) and boiled for 10 minutes in a microwave set at high. Sections were stained for 16 hours at 4°C with polyclonal anti-ILK antibody in 1% normal goat serum. Sections were then incubated with biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at 1:400 for 1 hour at room temperature. Avidin-biotin-peroxidase reagent (Vectastain Elite ABC Kit; Vector Laboratories), followed by 3,3'-diaminobenzidine (DAB Substrate Kit for Peroxidase; Vector Laboratories) was used for detection.

Statistical Analysis

Statistical analysis was performed using SigmaStat software (Systat Software Inc., San Jose, CA). Group means were compared by analysis of variance followed by Tukey's post hoc test for pairwise comparisons. For the SMC wound closure assay, a repeated measures analysis of variance was performed to compare the group means at each time point. For the cell attachment, cell proliferation, and fibronectin assembly assays, means were compared by student's *t*-test. Statistical significance was set at *P* < 0.05.

Results

Silencing ILK in Cultured SMC Potentiated SMC Wound Closure, Decreased Cell Attachment to Fibronectin, and Attenuated the Extracellular Assembly of Fibronectin Fibers

Western blots showed nearly complete inhibition of ILK protein expression after cells were transfected with targeted siRNA (Figure 1A), and there was no evidence for knockdown of unintended targets (eg, β -actin, Akt or GSK-3 β) (refer to Figure 3C, below; data not shown). Wound closure was significantly accelerated in ILK-silenced cells compared with untransfected or control siRNA transfected cells at 24 hours after wounding (Figure 1B). However the wounds closed in all groups by 60 hours, suggesting that the presence of ILK delayed but did not prevent wound closure. Since wound closure is the result of both migration and proliferation of SMCs, we also studied the effect of ILK-silencing on cell proliferation. Cell proliferation was increased by 1.8-fold in ILK-silenced cells compared with control siRNA transfected cells at 24 hours (*P* < 0.017). Taken together, these data

Figure 1. Silencing ILK increases the rate of wound closure. **A:** Western blot of cell lysates taken 60 hours after wounding, comparing ILK expression in untransfected SMCs, SMCs transfected with control siRNA, or with ILK siRNA. **B:** Wound closure assay with percent wound closure measured every 12 hours for 60 hours after wounding. *Significant difference between ILK-silenced cells and untransfected cells ($P < 0.05$). †Significant difference between ILK-silenced cells and control-siRNA transfected cells ($P < 0.05$). **C:** Attachment to fibronectin coated plates of control siRNA-transfected and ILK siRNA-transfected cells. *Significant difference between groups ($P < 0.05$). **D:** Fibronectin assembly is reduced in ILK-silenced SMCs. Oregon green-labeled soluble fibronectin protomers were incorporated into fibrils by control siRNA treated SMCs, but assembly was markedly reduced in SMCs treated with ILK siRNA.

suggest that increased proliferation along with increased cell migration contributed to the acceleration of wound closure.

Tight control of adhesive forces is necessary for optimum cell migration; accordingly, strong adhesion can prevent or delay migration.²² We assayed the adhesion of control and ILK-silenced SMCs to fibronectin-coated plates. Silencing ILK expression significantly decreased cell attachment by 33% (Figure 1C). Because ILK mediates fibronectin fibrillogenesis in fibroblasts,^{23,24} as further evidence of ILK functional knockdown, we measured fibrillogenesis in SMCs by incubating Oregon-green-labeled fibronectin protomers on a layer of SMCs *in vitro*. Silencing ILK decreased the assembly of fibronectin fibrils on the SMC surface (Figure 1D). Results were quantified by image analysis, and the fraction of the microscopic field occupied by fibronectin fibrils was reduced from $11.15 \pm 1.89\%$ in control cultures, to $4.37 \pm 0.86\%$ after siRNA treatment to reduce ILK ($\sim 60\%$ reduction, $P < 0.05$).

ILK Was Localized at Focal Adhesions in Vascular Smooth Muscle Cells

ILK can act as a scaffolding protein at focal adhesion sites, linking integrins to the actin cytoskeleton, which could impact on cell migration, therefore the localization of ILK in SMCs was determined. In subconfluent cell cultures, ILK immunostaining appeared in distinct dashes along the basal cell surface and was concentrated at the outer margins of the cell and in the protrusions of cell cytoplasm (Figure 2A). This ILK immunostaining colocalized with paxillin, a marker of focal adhesions (Figure 2, B and C, merged image). Treating cells with ILK siRNA resulted in the complete loss of immunostaining for ILK (Figure 2D). The intensity of paxillin staining was not affected in ILK-silenced cells, however the focal adhesions appeared to be longer and spread along the length of the cell (Figure 2, E and F). Double staining for ILK and actin was also performed. In control cells, ILK localized to the ends of actin stress fibers at the periphery of cells, and in parallel with actin stress fibers in the central portion of the cell (Figure 2, G–I). Silencing ILK resulted in the loss of staining for ILK (Figure 2J), and a modest decrease in central actin stress fibers (Figure 2, K and L).

ILK Did Not Regulate Akt or GSK3 β Phosphorylation During Wound Repair

The expression and phosphorylation of potential downstream substrates of ILK was assessed in wounded SMC cultures (Figure 3). In confluent cell cultures, phospho-Akt was undetectable, but after wounding phospho-Akt levels increased to a peak at 5 minutes with a 9.7-fold increase, then returned to undetectable levels at 4 hours after wounding (Figure 3A). Inhibitory phosphorylation of GSK3 β was low in confluent cells, but GSK3 β phosphorylation increased to a maximum (fivefold in-

Figure 2. ILK is localized to focal adhesions and the ends of actin stress fibers. **A–F:** Double immunostaining of subconfluent SMCs for ILK (red), paxillin (green) and nuclei (blue). Control siRNA treated cells stained for ILK (**A**), paxillin (**B**), and merged image (**C**). ILK siRNA-treated cells stained for ILK (**D**), paxillin (**E**), and merged image (**F**). **G–L:** Double staining of ILK (green), actin (red), and nuclei (blue). Control siRNA-treated cells stained for ILK (**G**), actin (**H**), and merged image (**I**). ILK siRNA-treated cells stained for ILK (**J**), actin (**K**), and merged image (**L**). Scale bars = 20 μ m.

crease) at 5 minutes after wounding, followed by a return to baseline levels at 24 hours (Figure 3B). There were no changes in total Akt or total GSK3 β levels after wounding.

The effects of silencing ILK are shown in Figure 3C. Transfection of targeting siRNA nearly abrogated ILK expression (reduction >90%), whereas the control siRNA increased ILK expression. β -actin expression was not affected by the siRNA. Silencing ILK did not affect the increases in phospho-Akt and phospho-GSK3 β that occurred after wounding (Figure 3C). There were no changes in total Akt or GSK-3 β after injury or ILK-silencing (data not shown).

ILK Protein Levels Were Decreased Following Balloon Catheter Injury of the Rat Carotid Artery

A marked and significant decrease in ILK was observed in arterial lysates after balloon catheter injury of the rat carotid artery (Figure 4A). The decrease in ILK was not due to the removal of endothelial cells by ballooning, because levels of ILK were equivalent in arterial lysates from uninjured vessels and vessels harvested at 5 minutes after injury (data not shown). Densitometric quantification revealed that ILK protein levels were significantly decreased at all time points after injury (Figure 4B). To

Figure 3. Wounding SMCs results in transient increases in phospho-Akt and phospho-GSK3 β that are not affected by ILK silencing. **A:** Representative immunoblots of lysates from unwounded (UI) and wounded SMCs after injury, probed with antibodies against phospho-Akt or total Akt. Densitometric analysis of phospho-Akt expression revealed a significant increase at 5 minutes after wounding. Values were normalized to total Akt to control for loading, and expressed relative to the uninjured control. **B:** Representative immunoblots of cell lysates from unwounded (UI) and wounded SMCs probed for phospho-GSK3 β and total GSK3 β . Densitometric analysis revealed a significant increase at 5 minutes after injury. Values were normalized to total GSK3 β to control for loading, and expressed relative to the uninjured control. **C:** Phosphorylation of Akt and GSK3 β is not dependent on ILK in wounded SMCs. Top panel: Western blot containing cell lysates, and probed for ILK, shows effective down-regulation of ILK expression by siRNA; second and third panels show Western blots probed for phospho-Akt and phospho-GSK3 β , and reveal increases in phosphorylation after wounding. Silencing ILK did not inhibit the phosphorylation of either mediator. Blots were stripped and re-probed with β -actin to demonstrate equal loading. The numbers underneath each panel represent the band density in that lane expressed as a fold change relative to the band in uninjured, untransfected cells.

measure levels of ILK in intimal versus medial layers of the vessel, the intimal layer was allowed to thicken for 14 days after injury, then it was stripped from the media, and separate intimal and medial lysates were probed for ILK on Western blots. The intimal layer of injured arteries contained more ILK than the medial layer (Figure 4C); however, ILK levels in both the intima and the media after injury were still significantly lower than levels in uninjured arteries.

ILK Expression and Activity Were Increased in Intimal SMCs at the Luminal Edge

ILK activity was detectable in uninjured carotid arteries, and appeared to decrease in carotids taken 14 days after injury (Figure 4D, upper panel). However, ILK protein content in the 14-day arteries was also decreased (Figure 4D, middle panel) and, when ILK activity was normalized to ILK protein content, there was a fivefold increase in ILK activity per unit of protein in the 14-day injured arteries (Figure 4D, lower panel). These findings suggested that ILK localized in the intima was active.

In normal uninjured vessels, immunostaining for ILK was evident in endothelial cells and SMCs throughout the media, as well as in scattered adventitial cells (Figure 5A). At 2 days after injury, complete denudation of the endothelial cells and a substantial loss of medial SMCs was evident (Figure 5B), consistent with previous reports using this injury model.^{25,26} ILK staining was decreased in the SMCs of the media at 2 days after injury (Figure 5B), and the medial staining remained low 7 and 14 days after injury (Figure 5, C and D). Despite the decrease in ILK staining in the media and the decrease in total ILK protein levels in tissue lysates, but in concurrence with the relatively higher levels of ILK in the intimal lysates on Western blots, substantial immunostaining for ILK was detected in the SMCs of the intima closest to the lumen at 7 and 14 days after injury (Figure 5, C and D); negative control is shown in Figure 5E.

Discussion

The current study provides evidence that ILK is robustly expressed in the quiescent arterial wall. ILK levels were

Figure 4. Balloon injury results in a decrease in ILK level in the arterial lysates. **A:** Representative Western blot of lysates from uninjured arteries (UI) and arteries at 2, 4, 7, and 14 days after injury probed with an antibody against ILK. A single band corresponding to the reported molecular weight of ILK is evident in all lanes. Blots were stripped and reprobed with an antibody against Akt to demonstrate even loading. **B:** Densitometric analysis of ILK expression revealed significant decreases in ILK at all times after injury. * $P < 0.05$ compared with uninjured arteries. **C:** ILK protein expression is increased in the intima relative to the media after vascular injury. Representative Western blot of ILK in lysates from uninjured control arteries (UI), and in medial and intimal lysates of arteries at 14 days after injury. Densitometric analysis revealed significant differences between lysates from media or intima after injury, compared with the uninjured control. * $P < 0.05$. **D:** Arterial injury up-regulates ILK kinase activity. The **top panel** includes an autoradiograph showing ILK kinase activity in uninjured carotid arteries (UI) and in carotid arteries 14 days after injury. The **middle** and **bottom panels** show Western blots of the same arterial lysates probed for ILK and β -actin, respectively. Densitometric analysis of labeled MBP normalized to total ILK protein level reveals an up-regulation of ILK activity per unit ILK protein.

dramatically suppressed during the phase of SMC migration and proliferation that followed balloon catheter injury *in vivo*; furthermore, ILK knockdown *in vitro* caused an increase in the rate of cell proliferation and wound closure by SMCs. Thus our data suggest that the decrease in ILK after injury permits SMC migration, proliferation, and neointimal thickening.

Because both proliferation and migration were increased in ILK-silenced cells, we attribute the acceleration of wound closure to these processes. We observed decreased attachment of ILK-silenced cells to a fibronectin matrix. The alterations in adhesion may have facilitated cell migration, because strong adhesion prevents migration, whereas intermediate levels of adhesion promote migration.²² The increased migration of SMCs after inhibition of ILK is consistent with a previous study showing that overexpression of wild-type ILK inhibited the polarization and migration of osteosarcoma cells, whereas overexpression of kinase-dead ILK, which did not localize to focal adhesions, resulted in an increase in cell migration.²⁷ One limitation of our studies is that the inhibition of ILK resulted in fairly modest alterations in wound closure and cell adhesion to matrix. Other pathways contribute to the regulation of these behaviors in SMCs, with the most likely candidates being integrin signaling through focal adhesion kinase, and cross-talk with growth factor receptor tyrosine kinases.

Co-immunostaining for ILK and paxillin revealed that ILK was localized to focal adhesions in smooth muscle cells. This finding was consistent with the localization of ILK and paxillin shown previously in SMCs,²⁸ and in migrating endothelial cells.²⁹ ILK mediates the recruitment of α - and β -parvins and PINCH1 to the adhesion site, where the molecular complex regulates the stability of the focal complex.^{30–33} We observed longer focal

adhesions in ILK-silenced SMCs. Our data are consistent with inhibition of ILK leading to alterations in focal adhesion distribution, which in turn could impact on cell proliferation and migration. Another study has shown that increased ILK expression was coincident with markedly enhanced actin stress fiber formation in senescent cardiac fibroblasts from aged rats.³⁴ Our results are consistent, showing that ILK-silencing decreased the formation of actin stress fibers in SMCs.

Silencing ILK did not affect the wound-induced phosphorylation of the kinases, Akt and GSK3 β . ILK may regulate SMC migration and adhesion by phosphorylating other substrates; alternatively, the scaffolding functions of the protein may be more important for focal adhesion dynamics and remodeling. The latter interpretation is consistent with findings that phosphorylation of Akt and GSK3 β are normal in ILK-deficient chondrocytes³⁵ and fibroblasts.³⁶ Furthermore, increases in ILK expression and activity are decoupled from Akt and GSK3 β phosphorylation in hypertrophic human hearts and in mouse hearts transgenically engineered to overexpress ILK.¹³ Another recent study questions the importance of ILK kinase activity in several non-transformed cell types.³⁷ ILK can also regulate the activation of Rac and Cdc42 via ILK interaction with the parvins and α PIX in some cell types.^{38–40} We did not assess Rho GTPase activation in our cells, however it is possible that these mediators were affected by ILK silencing.

Substantial levels of ILK were expressed in uninjured rat carotid arteries, in agreement with a recent publication showing ILK immunostaining in the aorta of mice.⁴¹ The rapid decreases in ILK protein that we observed after balloon injury, suggest that ILK suppression is important for migratory/proliferative responses to wounding. Immunostaining revealed that ILK was decreased in the media

Figure 5. ILK immunostaining decreases within the media, but remains strong in SMCs near the lumen of injured rat carotid arteries. ILK immunostaining in the uninjured carotid artery (**A**), and at 2 days (**B**), 7 days (**C**), and 14 days (**D**), after injury. **E**: Representative photomicrograph of a rat carotid artery at 14 days after injury stained with secondary antibody alone (negative control). Scale bars = 50 μ m. Nuclei are counterstained with hematoxylin.

of the vessel following a time course that coincides with active SMC proliferation and migration from the media to the intima.²⁶ We propose that the decrease in ILK facilitates the rearrangement of focal adhesions, allowing more efficient migration and proliferation of SMCs during neointimal growth.

Despite the dramatic decrease in ILK protein levels in the injured vessel lysates, there was increased immunostaining for ILK in SMCs at the luminal edge of the neointima. Furthermore, we observed an increase in kinase activity of ILK after injury, suggesting that this subluminal depot of ILK was very active. When combined with our finding that ILK promotes fibronectin assembly by SMCs, these data may explain previous observations that the luminal surface of the neointima is an area of fibronectin assembly.²⁰ In addition, fibronectin can act in a feed-forward mechanism to stimulate ILK activity.⁴ We therefore propose that interactions between a maturing fibronectin matrix and ILK stabilize the luminal surface of the artery by suppressing cell migratory and proliferative responses. Thus, the up-regulation of ILK at the lumen edge may be a turn-off switch arresting neointimal hyperplasia.

Our results differ somewhat from investigations in which overexpression of wild-type ILK in SMCs led to increased cell migration in response to SDF-1 or angiotensin II, while kinase-dead mutants of ILK (E359K) suppressed SMC migration in Boyden chamber assays.⁴¹ There are several possible explanations for the difference in results between this study and ours. The previous study reported on the overexpression of exogenous ILK or kinase-dead mutants of ILK, whereas we have shown the effects of inhibiting endogenous ILK by siRNA. It is possible that ILK-induced quiescence of SMC requires tight regulation of the intracellular levels of ILK and that both its suppression and up-regulation promote cell mo-

tility. Alternatively, the pro-migratory effects of ILK may be specific to SDF-1 or angiotensin II stimulation, or to the Boyden chamber assay. In our assay, wound closure on a fixed substrate is stimulated by the removal of neighboring cells and serum in the media, which may elicit different migratory behaviors.

In conclusion, we propose that ILK helps to maintain SMC in a stationary phenotype in the normal vessel wall. Following arterial injury, decreased ILK expression allows SMC migration and proliferation that establishes a thickened neointima. At later times, fibronectin deposition at the luminal edge of the vessel leads to the up-regulation of ILK expression, which then increases the adhesion of SMCs to contribute to the arrest of cell migration and proliferation at this location.

Note Added in Proof

While this manuscript was under review, a paper was published reporting expression of ILK in the injured rat carotid artery and studying ILK signaling in saphenous vein SMCs.⁴² Our results are consistent with findings from this study in that immunostaining for ILK was increased in the intima of balloon injured rat carotid arteries and in the developing intima of saphenous vein segments maintained in organ culture. However, the authors did not measure the protein levels of ILK nor ILK activity as we have. In contrast to our work, these authors observed decreased proliferation and migration of saphenous vein SMCs after transfecting siRNA for ILK. However, they achieved only a 60% knockdown in ILK levels, there was substantial variation in the baseline levels of ILK expression in the cell cultures, and they studied responses on laminin-coated plates, making it difficult to draw direct comparisons with our work.

References

- Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppelino MG, Radeva G, Filmus J, Bell JC, Dedhar S: Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature* 1996, 379:91–96
- Wu C: The PINCH-ILK-parvin complexes: assembly, functions and regulation. *Biochim Biophys Acta* 2004, 1692:55–62
- Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S: Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *J Biol Chem* 2001, 276:27462–27469
- Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S: Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* 1998, 95:11211–11216
- Persad S, Dedhar S: The role of integrin-linked kinase (ILK) in cancer progression. *Cancer Metastasis Rev* 2003, 22:375–384
- Fielding AB, Dobrev I, McDonald PC, Foster LJ, Dedhar S: Integrin-linked kinase localizes to the centrosome and regulates mitotic spindle organization. *J Cell Biol* 2008, 180:681–689
- Oloumi A, McPhee T, Dedhar S: Regulation of E-cadherin expression and beta-catenin/Tcf transcriptional activity by the integrin-linked kinase. *Biochim Biophys Acta* 2004, 1691:1–15
- Friedrich EB, Liu E, Sinha S, Cook S, Milstone DS, MacRae CA, Mariotti M, Kuhlencordt PJ, Force T, Rosenzweig A, St-Arnaud R, Dedhar S, Gerszten RE: Integrin-linked kinase regulates endothelial cell survival and vascular development. *Mol Cell Biol* 2004, 24:8134–8144
- Vouret-Craviari V, Boulter E, Grall D, Matthews C, Van Obberghen-Schilling E: ILK is required for the assembly of matrix-forming adhesions and capillary morphogenesis in endothelial cells. *J Cell Sci* 2004, 117:4559–4569
- Kaneko Y, Kitazato K, Basaki Y: Integrin-linked kinase regulates vascular morphogenesis induced by vascular endothelial growth factor. *J Cell Sci* 2004, 117:407–415
- Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K, Wheeler J, Gleave M, Sanghera J, Dedhar S: Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* 2004, 5:79–90
- White DE, Coutu P, Shi YF, Tardif JC, Nattel S, St AR, Dedhar S, Muller WJ: Targeted ablation of ILK from the murine heart results in dilated cardiomyopathy and spontaneous heart failure. *Genes Dev* 2006, 20:2355–2360
- Lu H, Fedak PWM, Dai X, Du C, Zhou YQ, Henkelman M, Mongroo PS, Lau A, Yamabi H, Hinek A, Husain M, Hannigan G, Coles JG: Integrin-linked kinase expression is elevated in human cardiac hypertrophy and induces hypertrophy in transgenic mice. *Circulation* 2006, 114:2271–2279
- Hannigan GE, Coles JG, Dedhar S: Integrin-linked kinase at the heart of cardiac contractility, repair, and disease. *Circ Res* 2007, 100:1408–1414
- Deng JT, Van Lierop JE, Sutherland C, Walsh MP: Ca²⁺-independent smooth muscle contraction: a novel function for integrin-linked kinase. *J Biol Chem* 2001, 276:16365–16373
- Wilson DP, Sutherland C, Borman MA, Deng JT, Macdonald JA, Walsh MP: Integrin-linked kinase is responsible for Ca²⁺-independent myosin diphosphorylation and contraction of vascular smooth muscle. *Biochem J* 2005, 392:641–648
- Muranyi A, Macdonald JA, Deng JT, Wilson DP, Haystead TA, Walsh MP, Erdodi F, Kiss E, Wu Y, Hartshorne DJ: Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem J* 2002, 366:211–216
- Franco CD, Ho B, Mulholland D, Hou G, Donaldson KL, Bendeck MP: Doxycycline alters vascular smooth muscle cell adhesion, migration and reorganization of fibrillar collagen matrices. *Am J Pathol* 2006, 168:1697–1709
- Hou G, Vogel W, Bendeck MP: The discoidin domain-receptor tyrosine kinase DDR1 in arterial wound repair. *J Clin Invest* 2001, 107:727–735
- Pickering JG, Chow LH, Li S, Rogers KA, Rocnik EF, Zhong R, Chan BMC: [alpha]5[beta]1 integrin expression and luminal edge fibronectin matrix assembly by smooth muscle cells after arterial injury. *Am J Pathol* 2000, 156:453–465
- Bendeck MP, Conte M, Zhang M, Nili N, Strauss BH, Farwell SM: Doxycycline modulates smooth muscle cell growth, migration and matrix remodeling after arterial injury. *Am J Pathol* 2002, 160:1089–1095
- Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF: Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 1997, 385:537–540
- Wu C, Keightley SY, Leung-Hagesteijn C, Radeva G, Coppelino M, Goicoechea S, McDonald JA, Dedhar S: Integrin-linked protein kinase regulates fibronectin matrix assembly. E-cadherin expression, and tumorigenicity *J Biol Chem* 1998, 273:528–536
- Kagami S, Shimizu M, Kondo S, Kitamura A, Urushihara M, Takamatsu M, Yamaji S, Ishigatsubo Y, Kawachi H, Shimizu F: Up-regulation of integrin-linked kinase activity in rat mesangiolipofibroblasts. *Life Sci* 2006, 78:1794–1800
- Lindner V, Reidy MA: Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci USA* 1991, 88:3739–3743
- Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium *Lab Invest* 1983, 49:327–333
- Khyrul WA, Lalonde DP, Brown MC, Levinson H, Turner CE: The integrin-linked kinase regulates cell morphology and motility in a ROCK-dependent manner. *J Biol Chem* 2004, 279:54131–54139
- Nikolopoulos SN, Turner CE: Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions. *J Biol Chem* 2001, 276:23499–23505
- Joshi MB, Ivanov D, Philippova M, Erne P, Resink TJ: Integrin-linked kinase is an essential mediator for T-cadherin-dependent signaling via Akt and GSK3beta in endothelial cells. *FASEB J* 2007, 21:3083–3095
- Zhang Y, Chen K, Tu Y, Velyvis A, Yang Y, Qin J, Wu C: Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites. *J Cell Sci* 2002, 115:4777–4786
- Fukuda T, Chen K, Shi X, Wu C: PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. *J Biol Chem* 2003, 278:51324–51333
- Tu Y, Huang Y, Zhang Y, Hua Y, Wu C: A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. *J Cell Biol* 2001, 153:585–598
- Rosenberger G, Jantke I, Gal A, Kutsche K: Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. *Hum Mol Genet* 2003, 12:155–167
- Chen X, Li Z, Feng Z, Wang J, Ouyang C, Liu W, Fu B, Cai G, Wu C, Wei R, Wu D, Hong Q: Integrin-linked kinase induces both senescence-associated alterations and extracellular fibronectin assembly in aging cardiac fibroblasts. *J Gerontol A Biol Sci Med Sci* 2006, 61:1232–1245
- Grashoff C, Aszodi A, Sakai T, Hunziker EB, Fassler R: Integrin-linked kinase regulates chondrocyte shape and proliferation. *EMBO Rep* 2003, 4:432–438
- Sakai T, Li S, Docheva D, Grashoff C, Sakai K, Kostka G, Braun A, Pfeifer A, Yurchenco PD, Fassler R: Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev* 2003, 17:926–940
- Troussard AA, McDonald PC, Wederell ED, Mawji NM, Filipenko NR, Gellman KA, Kucab JE, Dunn SE, Emerman JT, Bally MB, Dedhar S: Preferential dependence of breast cancer cells versus normal cells on integrin-linked kinase for protein kinase B/Akt activation and cell survival. *Cancer Res* 2006, 66:393–403
- Zhang Y, Chen K, Tu Y, Wu C: Distinct roles of two structurally closely related focal adhesion proteins, alpha-parvins and beta-parvins, in regulation of cell morphology and survival. *J Biol Chem* 2004, 279:41695–41705
- Filipenko NR, Attwell S, Roskelley C, Dedhar S: Integrin-linked kinase activity regulates Rac- and Cdc42-mediated actin cytoskeleton reorganization via alpha-PIX. *Oncogene* 2005, 24:5837–5849
- Mishima W, Suzuki A, Yamaji S, Yoshimi R, Ueda A, Kaneko T,

- Tanaka J, Miwa Y, Ohno S, Ishigatsubo Y: The first CH domain of affixin activates Cdc42 and Rac1 through alphaPIX, a Cdc42/Rac1-specific guanine nucleotide exchanging factor. *Genes Cells* 2004, 9:193-204
41. Friedrich EB, Clever YP, Wassmann S, Werner N, Bohm M, Nickenig G: Role of integrin-linked kinase in vascular smooth muscle cells: regulation by statins and angiotensin II. *Biochem Biophys Res Commun* 2006, 349:883-889
42. Dwivedi A, Sala-Newby GB, George SJ: Regulation of cell-matrix contacts and beta-catenin signaling in VSMC by integrin-linked kinase: implications for intimal thickening. *Basic Res Cardiol* 2007, 103:244-256