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

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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 β1- and β3-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 3β (GSK3β) 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 development8,9 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-
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-GSK3β (Ser9) (#9336). The monoclonal anti-GSK3β antibody (clone 4G-1E), and the polyclonal anti-ILK antibody (#06-592) used for immunoprecipitation were from Upstate Cell Signaling Solutions. Rabbit polyclonal anti-ILK antibodies (clone 4G-1E), and the polyclonal anti-ILK antibody (NM_133409). For each well, 90 pmol of either 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 μl Opti-MEM I reduced serum medium and 5 μl 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 concentration 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/ml Alamar Blue (Biosource, Camarillo, CA) 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 precoated with 10 nmol/L fibronectin as previously described. 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 Hoescht 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-3β 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 × 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 μl lysis buffer, and the lysates were used for Western blotting to measure phospho-Akt, Akt, phospho-GSK3β, GSK3β, and β-actin.

siRNA was also used to determine whether ILK mediated the phosphorylation of Akt and GSK3β 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 μl 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-GSK3β, and β-actin.

Western Blotting

Western blots containing cell lysates (10 μg total protein per lane) were incubated for 16 hours at 4°C with anti-ILK, anti-phospho-Akt, anti-Akt, anti-phospho-GSK3β, 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-GSK3β were normalized to β-actin, total Akt and GSK3β, 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, Rompun; 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. 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 μl lysis buffer, composed of 34.7 M SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 23.4 μmol/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 2x sample buffer composed of 0.1 M Tris pH 6.8, 69.4 mmol/L SDS, 20% glycerol and 0.1% bromphenol blue. Native SDS-polyacrylamide gels were loaded with 10 μg 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 to 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 ± 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 μl 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 μl 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 μmol/L leupeptin, 154 nmol/L aprotinin, and 3 mmol/L phenylmethylsulfonyl fluoride. ILK was immunoprecipitated from 160 μg of total protein from arterial lysates by incubating with 1.44 μg rabbit polyclonal anti-ILK antibody (Upstate Cell Signaling) at 4°C for 16 hours. Immune complexes were collected in 50 μl of 50% protein A agarose in NP40 lysis buffer, rotated at 4°C for 2 hours, then washed with NP-40 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 μl 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 μg MBP and 5 μCi [γ³²P] ATP. After a 30-minute incubation at 37°C, the reaction was terminated with 8 μl of 5X SDS sample buffer; 35 μl 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 μm-thick cross-sections. Sections were deparaffinized in xylene, 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-3β) (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...
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. 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 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 ± 1.89% in control cultures, to 4.37 ± 0.86% after siRNA treatment to reduce ILK (~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 GSK3β 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 GSK3β was low in confluent cells, but GSK3β phosphorylation increased to a maximum (fivefold in-
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 GSK3β 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-GSK3β that occurred after wounding (Figure 3C). There were no changes in total Akt or GSK-3β 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

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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 μm.
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. 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
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. 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. 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, and in migrating endothelial cells. 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. 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. 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 GSK3β. 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 GSK3β are normal in ILK-deficient chondrocytes and fibroblasts. Furthermore, increases in ILK expression and activity are decoupled from Akt and GSK3β phosphorylation in hypertrophic human hearts and in mouse hearts transgenically engineered to overexpress ILK. Another recent study questions the importance of ILK kinase activity in several non-transformed cell types. ILK can also regulate the activation of Rac and Cdc42 via ILK interaction with the parvins and αPIX in some cell types. 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. 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.
of the vessel following a time course that coincides with active SMC proliferation and migration from the media to the intima. 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. In addition, fibronectin can act in a feed-forward mechanism to stimulate ILK activity. 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. 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 motility. 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. 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.
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