The Role of Integrin-linked Kinase in Liver Wound Healing*

Mahnoush S. Shafiei and Don C. Rockey

From the Division of Digestive and Liver Diseases, Department of Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8887

Liver wound healing is an integrated process in which hepatic stellate cells play a major role. We hypothesized that the cell-extracellular signaling protein integrin-linked kinase (ILK) is important in transducing signals from the extracellular matrix to stellate cells and thus plays a critical role in stellate cell activation and fibrogenesis during liver injury. Liver injury and subsequent stellate cell activation led to a 3-fold increase in ILK expression and increased kinase activity. Overexpression of ILK in isolated stellate cells led to enhanced motility and adhesion as well as increases in smooth muscle α-actin and type I collagen mRNA expression. The effects of ILK on stellate cell phenotypes were phosphatidylinositol 3-kinase-dependent. Forced expression of ILK in vivo led to increases in type I collagen, smooth muscle α-actin, transforming growth factor-β, and extra domain A (EDA) fibronectin mRNAs (by 3.2-, 3.5-, 2.5-, and 2.2-fold, respectively; n = 8, p < 0.05 for each versus the control), whereas inhibition of ILK in vivo led to significant reductions in these mRNAs. Morphometric analysis revealed that ILK overexpression led to a 31.4% increase in liver collagen content (n = 8, p < 0.05 versus the control); in contrast ILK knockdown in vivo led to a significant reduction in fibrogenesis. We conclude that ILK plays an important pathophysiological role in vivo in liver wound healing.

The liver responds to injury by wound healing, leading to fibrosis. The multiple integrated systems leading to fibrosis are complex and are similar mechanistically to that in skin and other parenchymal organs. A remarkable increase in extracellular matrix production after wounding is characteristic and is typified by a 4–6-fold increase in collagens (i.e. types I, III, and IV, etc.) as well as an increase in many other extracellular matrix constituents (1). Investigation over the past two decades has helped to establish a cellular mechanism for the wound-healing response to liver injury (2). A critical feature of this wounding response is the transformation of resident stellate cells (also known as lipocytes, Ito cells, or perisinusoidal cells) from the “quiescent” state in the normal liver to an “activated” state in the injured liver. This transition is characterized by both morphologic and functional changes, including loss of vitamin A, acquisition of stress bundles, development of prominent rough endoplasmic reticulum, and a striking increase in the secretion of extracellular matrix proteins, as well as enhanced stellate cell contractility, motility, proliferation, adhesion, and responsiveness to various cytokines and peptides found in the wounding milieu (3).

Integrin-linked kinase (ILK) is a newly described signaling molecule important in transducing signals from the extracellular matrix to cellular machinery through the cytoplasmic portion of integrins and adaptor proteins to a variety of signaling cascades (4–8). For example, ILK interaction with integrins is important in cell growth, differentiation, cell motility, and cell attachment (4–8). ILK adaptor function seems to be especially important for cell spreading, F-actin organization, and formation of focal contacts, as demonstrated by reconstitution experiments with kinase-dead ILK in ILK-deficient fibroblasts (9).

Interestingly, little is known about the regulation of ILK expression. However, ILK kinase activity is stimulated by growth factors and by attachment of cells to the extracellular matrix, (for example, to fibronectin) (10). Downstream of ILK, a number of effectors have been identified, including protein kinase B (PKB/Akt) and glycogen synthase kinase (GSK)-3β, and appear to be targets of ILK (11–13). Akt, in particular, appears to be important in mediating ILK effects (13, 14).

Given the critical role of ILK in mediating extracellular-intracellular signals and the exuberant extracellular matrix response typical of liver injury, we hypothesized that ILK is important in linking extracellular signals to stellate cells and therefore may mediate specific functional phenotypes in hepatic stellate cells, key effectors of the liver wounding response. Here, we have demonstrated, not only that ILK expression is up-regulated during liver injury but also that it has critical functional effects in isolated stellate cells as well as in vivo during wound healing.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse anti-ILK monoclonal antibody was purchased from BD Transduction Laboratories (Lexington, KY). Endothelin-1 (ET-1) and endothelin antagonists (BQ-123 and BQ-788) were from American Peptide Co. Inc. (Sunnyvale, CA). Recombinant TGF-β was from R&D Systems (Minneapolis, MN). The AdEasy™ XL adenoviral vector system was from Stratagene (La Jolla, CA). Secondary

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1 Recipient of a Burroughs Wellcome Fund Translational Scientist Award. To whom correspondence should be addressed: Div. of Digestive and Liver Diseases, University of Texas Southwestern Medical Ctr., 5323 Harry Hines Blvd, Dallas, TX 75390-8887. Tel.: 214-648-3444; Fax: 214-648-0274; E-mail: don.rockey@utsouthwestern.edu.

2 The abbreviations used are: ILK, integrin-linked kinase; PI, phosphatidylinositol; TGF, transforming growth factor; BDK, bile duct ligation; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Ad, adenovirus.-
ILK and Liver Fibrosis

a

![Image of ILK and β-actin immunoblots](Image)

b

![Image of Normal and Activated (BDL) ILK and β-actin immunoblots](Image)

c

![Image of ILK expression over time](Image)

FIGURE 1. Increased expression of ILK after liver injury and stellate cell activation. a, liver injury was induced by bile duct ligation (BDL) or carbon tetrachloride (CCl4) administration as described under “Experimental Procedures.” Stellate cells were isolated, and cell lysates (25 μg of total protein) were subjected to immunoblot to detect ILK and β-actin (upper panels). Below the representative, respective immunoblots, data were quantified, normalized to the signal for β-actin, and presented graphically (n = 5; *, p < 0.05 compared with normal animals). b, stellate cells isolated from normal or injured (BDL) rats were allowed to adhere to glass-coated culture dishes and grown in serum-containing medium for 1 day. Cells were fixed, and immunohistochemistry was performed as described under “Experimental Procedures.” Representative images are shown (ILK is labeled with AL-488 (green), and nuclei are labeled with DRAQ5 (blue)). The bar shown in the lower panel represents 10 microns. c, stellate cells from normal rats were isolated and grown on plastic culture dishes (in the presence of serum-containing medium) for the indicated number of days. At the indicated time points, lysates (25 μg of total protein) were subjected to immunoblotting with anti-ILK monoclonal antibody. A representative immunoblot is shown in the upper panel, and below it, a stripped blot reprobed for β-actin; subsequently, data from independent experiments were quantified, normalized to the signal for β-actin and presented graphically (n = 5; *, p < 0.05 compared with the signal for day 1). The positive control consists of lysate rich in ILK obtained from the manufacturer of the ILK antibody.

antibodies were from Amersham Biosciences. Culture media, fetal bovine serum, and supplements were obtained from Invitrogen. All other chemicals were of analytic grade and obtained from Sigma.

Cell Isolation and Culture—Male Sprague-Dawley rats (450–500 g) (Harlan, Indianapolis, IN) were maintained under temperature-controlled conditions (22 ± 2°C) in 12-h light/dark cycles with food and water ad libitum. All animals received humane care in compliance with the University of Texas Southwestern Medical Center Animal Care and Use Guidelines. Hepatic stellate cells were isolated by the combined digestion of the liver with Pronase (Roche Applied Science) followed by collagenase (Crescent Chemical, Hauppauge, NY). In brief, stellate cells were separated from other liver non-parenchymal cells by ultracentrifugation over gradients of 8.2 and 15.6% Accudenz (Accurate Chemical & Scientific, Westbury, NY). The resulting upper layer consisted of >95% stellate cells. Cells were plated on uncoated plastic and maintained in modified medium 199 containing 10% fetal bovine serum and 10% calf serum (Invitrogen) with antibiotics (penicillin, 100 units/ml; streptomycin, 100 units/ml; gentamicin, 0.1 mg/ml; and amphotericin B, 2.5 μg/ml). Cultures were incubated at 37°C in a humidified incubator (containing 95% O2 and 2.5% CO2), and the medium was changed every 24 h. Cell viability was >90% in all cultures used. The cells were considered to be quiescent at day 1, 24 h after plating. Cells at day 7 were considered activated, with >95% staining positive for smooth muscle actin. For all experiments in which cells at different stages were compared, the cells were from the same isolation. All experiments were repeated with cells from different animals.

Animal Models of Liver Injury—Liver injury was induced by the administration of carbon tetrachloride (CCl4) or by performing bile duct ligation (BDL) in 450–500-g male retired breeder Sprague-Dawley rats as described previously (15, 16). BDL resulted in periportal expansion of the biliary duct cells with concomitant periductular and lobular extracellular matrix deposition. In sham-operated rats, an incision was made in the abdomen, which was then closed without any treatment. Carbon tetrachloride administration led to central-central and central-portal extracel-
TTT CCA ACT CGA G-3’ and reverse, 5’-CGC GTC ATT GGT GCC TTC ATG TAG AAC ACC ACA CCT GTT CTA CAT GAA GGC ACC AAT TTT TTC CAA C-3’. Control sequences included the following: scrambled sequence forward, 5’-CGC GTC ACA CTA TCC TGT ATC GGC CTC TAG AGC GCC ATA CAG GAT AGT TTT TCC AAC-3’ and reverse, 5’-AGT GTG ATA GGA CAT AGC GCG ATA TCT CGC GCT ATG ACC TAT CAC AAA AAA GGT TGA CTT-3'; mismatch sequence forward, 5’-CGC GTC ACC CCT TAC AGA ATG AGT CCT TCC ACA CCA AGC ACT CAT TCT GTA AGG GGT TTT TTC CAA C-3’ and reverse, 5’-AGT GGG GAA TGT CTT ACT CAG GAA GGT GTG TCT CCT GAG TAA GAC ATT CCC CAA AAA AGG TTG AGC T-3’; and nonspecific sequence forward, 5’-CGC GTC AAA TTG ATG TGT TTA GTC GCT ACC and reverse, 5’-AGT TTA ACT ACA CAA ATC AGG GGT GTG GAT CGC TGA TTT GTG TAG TTA AAA AA A AGG TTG AGC T-3’. Transient transfection of LX2 cells was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Oligonucleotides were annealed and ligated to the MluI and XhoI sites of Ad-Track, and the inserted sequences were confirmed by sequencing.

**Immunoblotting**—Cell lysates were prepared in a buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 50 mM NaF, 50 mM sodium-2-glycerophosphate, 0.05 mM Na3VO4, 10 μg/leupeptin, 10% glycerol, and 100 mM phenylmethylsulfonyl fluoride. Samples containing 50 μg of total protein were subjected to SDS-PAGE, after which proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated for 1 h at room temperature in blocking buffer (10 mM sodium phosphate, 0.5 mM NaCl, 0.05% Tween 20, and 2.5% dry milk) and then with primary antibody (1:1000) overnight at 4 °C. Membranes were then washed of excess primary antibody at room temperature in a phosphate-buffered saline Tween buffer (TBST: 10 mM NaCl, 0.05%, Tris pH 8, 0.9% sodium chloride, Tween 20 0.05% dry milk) and then with secondary antibody. Blots were stripped and probed for α-tubulin (shown in the upper panel). Total ILK was detected using specific sequences included the following: scrambled sequence forward, 5’-CGC GTC ACA CTA TCC TGT ATC GGC CTC and reverse, 5’-AGT GTG ATA GGA CAT AGC GCG ATA TCT CGC GCT ATG ACC TAT CAC AAA AAA GGT TGA CTT-3’. Transient transfection of LX2 cells was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Oligonucleotides were annealed and ligated to the MluI and XhoI sites of Ad-Track, and the inserted sequences were confirmed by sequencing.

**Kinase Activity**—Kinase assays were carried out as previously described (17). In brief, cell lysates were immunoprecipitated overnight at 4 °C with anti-ILK antibody. The solution was centrifuged, and immunoprecipitates were washed three times with cell lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 10 mM MgCl2, 0.1 mM Na3VO4, and 2 mM dithiothreitol). Immunoprecipitated proteins were incubated in 20 μl of kinase buffer containing 1 μg of myelin-basic protein and [γ-32P]ATP (10 μCi) for 30 min at 30 °C. The phosphorylated substrate was then resolved by SDS-PAGE followed by autoradiography.

**Immunofluorescence Microscopy**—Hepatic stellate cells were isolated, placed on glass-bottomed plastic culture
ILK and Liver Fibrosis

FIGURE 3. Regulation of ILK expression. a, stellate cells were isolated and allowed to undergo culture-induced activation for 7 days as described in the legend to Fig. 1. Cells were then serum-starved overnight and exposed to ET-1 at the indicated concentrations for 48 h. Cell lysates were immunblotted to detect ILK as described under “Experimental Procedures.” A representative immunoblot is shown in the upper panel of a, and in the middle panel, a blot stripped and reprobed to detect β-actin. In the lower panel, data from independent experiments were quantified, normalized to the signal for β-actin, and presented graphically (n = 5; *, p < 0.05 versus no ET-1). b, stellate cells were processed as described for a and were subsequently exposed to 20 nM ET-1 for 24 h in the absence or presence of 20 nM E7394 (ETα antagonist), BQ-788 (ETβ antagonist), or both. In the upper panel of b, a representative immunoblot is shown (ILK, top panel, and β-actin, middle panel), and in the bottom panel, data were quantified, normalized to the signal for β-actin, and presented graphically (n = 5; *, p < 0.05 versus control). c, stellate cells, as described for a, were serum-starved overnight and exposed to the indicated concentrations of TGF-β for 48 h. In the upper panel of c, a representative immunoblot is shown, and in the middle panel, a blot stripped and reprobed to detect β-actin; below it, data from independent experiments were quantified, normalized to the signal for β-actin, and presented graphically (n = 5; *, p < 0.05 versus no TGF-β).

dishes and allowed to undergo activation as described above. For detection of ILK, the cells were fixed in 50% acetone/methanol, washed, and exposed to monoclonal anti-ILK antibody (1:1,000) in PBS. After washing, primary antibody was detected with Alexa Fluor® 488 (Molecular Probes)-conjugated anti-mouse and DRAQ5 (Alexis Corp., San Diego, CA) to identify the nuclei. After washing and mounting, signals were visualized with a Zeiss LSM 510 META confocal microscope. A solution of bovine serum albumin/PBS at 3% and a non-immune mouse IgG were employed as a negative control.

Cell Migration Assay—Cell migration was assessed by measuring the repair of a linear wound generated in the confluent monolayer of cells. Hepatic stellate cells were isolated, plated at an equivalent density on chamber slides (Lab-Tek, Westmont, IL), and allowed to undergo culture-induced activation for 7 days. They were then infected with constructs (all at a multiplicity of infection of 100) or went untreated for 24 h. Some cells were exposed to wortmannin (200 nM) for 20 min prior to the application of a linear scratch in cell monolayers using a sterile plastic pipette tip. Cell migration was recorded for 24 h using a Zeiss Axiovert 200 multidimensional time lapse work station.

Cell Adhesion Assay—Hepatic stellate cells were isolated, plated at an equivalent density, and allowed to undergo culture-induced activation for 7 days. They were then infected with specified adenoviruses (all at a multiplicity of infection of 100) or went untreated. Forty-eight hours after viral infection, the cells were detached from the culture dishes with trypsin. Some cells were exposed to wortmannin (200 nM). The detached cells were suspended in Dulbecco’s modified Eagle’s medium, 20% serum was added, and the cells were then replated on fresh tissue culture dishes. Two hours after replating, the cells were washed twice with PBS to remove unattached cells, trypsinized, and the adherent cells were counted.

Morphometry—Livers were fixed in 10% phosphate-buffered formalin for 48 h at 4 °C, washed twice with water, stored in 70% ethanol at 4 °C for 24 h, and then embedded in paraffin. Five-micron sections were then dehydrated and stained with 0.1% sirius red F3B in saturated picric acid and counterstained with fast green (all from Sigma). The proportion of tissue stained with picrosirius red content was assessed by morphometric analysis using MetaView software (Universal Imaging Corp, Downingtown, PA); collagen stained with sirius red was quantitated in the sections that were randomly chosen (under 20× magnification, 10 fields each from sample).

Hydroxyproline Assay—Liver tissue was weighed, hydrolyzed in 6 N HCl, and incubated at 110 °C for 16 h, filtered, and then 25 μl of the samples were freeze-dried for 4 h. The sediment was redissolved in 50% isopropyl alcohol and then incubated with 0.5 ml of chloramine-T solution containing 1.41 g of chloram-
ine-T, dissolved in 12% Ehrlich’s reagent, and then incubated at room temperature for 10 min. To this were added 0.5 ml of 12% Ehrlich’s solution, including 10 g of dimethylaminobenzaldehyde dissolved in 11 ml of 60% perchloric acid, and the mixture was incubated at 50 °C for 90 min. After cooling, the absorbance was read at 558 nm. Hydroxyproline concentration was calculated from a standard curve prepared with high purity hydroxyproline (Sigma) and expressed as mg of hydroxyproline/mg of liver.

Statistical Analysis—Results are expressed as mean ± S.E. Significance was established using the Student’s t test and analysis of variance when appropriate. Differences were considered significant when p < 0.05.

RESULTS

In Vivo Up-regulation of ILK Expression after Injury and Wound Healing—To examine the expression of ILK in vivo during wound healing, liver injury created by BDL, or by exposure of rats to repetitive doses of CCl₄, stellate cells were subsequently isolated and immediately subjected immunoblotting to detect ILK. Regardless of the type of liver injury, ILK expression was significantly up-regulated (Fig. 1a). We further examined ILK expression using immunohistochemistry; stellate cells isolated from normal rats expressed relatively small amounts of ILK (i.e. at day 1 after isolation, cells were small, and the ILK found within the cells remained disorganized), whereas cells isolated from injured rats, not only took on an activated appearance (activated cells spread over time becoming larger in size), but they also expressed larger quantities of ILK, which was identified in focal adhesions (Fig. 1b). Additionally, in a culture model that mimics stellate cell activation after liver injury, we found that ILK expression appeared to increase sequentially in proportion to the degree of stellate cell activation. In this model, stellate cell activation proceeds in early culture through day 14 in culture. ILK expression was detected early, increased markedly at day 4, and peaked after 14 days (Fig. 1c). Additionally, we found that ILK activation paralleled ILK expression (Fig. 2). In kinase assays shown in Fig. 2, kinase activity became increased as ILK was up-regulated.

ILK Expression Is Regulated by Factors Found in the Wounding Environment—The wounding response is characterized by production of cytokines and a variety of peptides that help drive the wounding response. Therefore, we sought to determine whether two typical compounds found in the wounding environment, ET-1 and TGF-β, might regulate expression of ILK. In these experiments, hepatic stellate cells were specifically stimulated with ET-1 and TGF-β. We found that both compounds potently induced ILK expression (Fig. 3). Interestingly, we found that stimulation of either the ETₐ or ETₐ receptor mediated the up-regulation of ILK, and blockade of both receptors had a slightly additive effect (Fig. 3b), suggesting that each receptor is capable of signaling to ILK regulatory pathways. Interestingly, other compounds found in the wounding milieu, such as FGF, did not lead to up-regulation of ILK (data not shown).

Modulation of ILK Expression—To better understand the functional role of ILK in stellate cells in liver wound healing, we developed constructs with which to reduce (an adenovirus expressing ILK small interfering short hairpin RNA, Ad-shILK) or to overexpress (an adenovirus expressing ILK, Ad-ILK) ILK selectively in infected cells. Constructs were found to both knock down (Fig. 4, a and b) and overexpress ILK (Fig. 4b).
FIGURE 5. ILK modulates stellate cell adhesion and migration. a, stellate cells were isolated, plated at equivalent density, allowed to undergo culture-induced activation for 7 days as described in the legend to Fig. 1, and infected with the indicated adenoviral constructs (adenovirus-expressing ILK (Ad-ILK); adeno virus-expressing ILK small interfering short hairpin RNA (Ad-shILK), the construct used corresponds to that shown in Fig. 4b) or a matched control adenovirus-expressing GFP (Ad-GFP) as described under “Experimental Procedures.” Forty-eight hours later (in some cultures, wortmannin (200 nM) was added 20 min prior to cell detachment), the cells were detached and resuspended in Dulbecco’s modified Eagle’s medium with 20% serum, and then replated as described under “Experimental Procedures.” Two hours later, the cells were washed twice with PBS to remove the unattached cells, and the remaining cells were counted (n=3; *, p<0.05 versus control; #, p<0.01 versus Ad-GFP; †, p<0.05 versus Ad-ILK). b, stellate cells were isolated, plated, and infected with constructs as shown above in this figure, and when indicated, wortmannin was added, as described for a; 48 h after infection, a wound was created on confluent cell monolayers as described under “Experimental Procedures,” and the distance that the wound edge migrated was assessed for 24 h using time lapse microscopy at 37 °C as described under “Experimental Procedures” (n=3; *, p<0.05 versus control; #, p<0.01 versus Ad-GFP; †, p<0.05 versus Ad-ILK). c–f, culture-activated immunohistochemistry of stellate cells was performed. c shows a cell exposed to serum, and d, serum-free medium. Cells were infected with adenoviral constructs as described above: Ad-ILK (e), Ad-shILK (f); 48 h later, the cells were fixed, and actin stress fibers were visualized as described under “Experimental Procedures.” The scale bar shown in the lower right of f represents 5 microns. g, culture-activated stellate cells were transfected to serum-free conditions, infected with adenoviral constructs as described above, and in some cultures, serum (an amount to yield a total of 10% bovine + 10% horse) or wortmannin (200 nM) was added; 48 h later, RNA was harvested and subjected to reverse transcription-PCR to detect type I collagen mRNA as described under “Experimental Procedures” (n=3; *, p<0.05 versus control; #, p<0.01 versus Ad-GFP; †, p<0.05 versus Ad-ILK). h, culture-activated stellate cells, grown in serum-containing medium as described under “Experimental Procedures,” were infected with the indicated adenoviral constructs as described above, and in certain cultures, wortmannin was added (200 nM); 48 h later, cell lysates (20 μg of total protein) were subjected to immunoblotting smooth muscle α-actin (Sm α-actin) as described under “Experimental Procedures” (upper panel); in the middle panel, a blot was stripped and reprobed to detect β-actin, and in the lower panel, data from independent experiments were quantified, normalized to the signal for β-actin, and presented graphically (n=3; *, p<0.05 versus control; #, p<0.01 versus Ad-GFP; †, p<0.05 versus Ad-ILK).
ILK and Liver Fibrosis

Another important phenotype for mesenchymal cells, such as stellate cells, during wound healing is cell migration. Indeed, migration of stellate cells appears to be one of their most prominent phenotypes (18). Therefore, we tested whether ILK could mediate stellate cell migration. In an assay system that allows assessment of cellular migration, we found that overexpression of ILK led to a marked increase in stellate cell migration, whereas inhibition of ILK led to reduced cell migration (Fig. 5b). Migration, as cell adhesion, appeared to be PI 3-kinase-dependent, because exposure of cells to wortmannin inhibited the effect of overexpression of ILK.

We next examined the effect of ILK on the stellate cell cytoskeleton (Fig. 5, c–f). We found that serum induced prominent stress (Fig. 5c), whereas exposure of stellate cells to serum-free medium led to a slight disassembly of the cytoskeleton (Fig. 5d). Interestingly, overexpression of ILK led to an even greater prominence of the cytoskeleton (Fig. 5e), whereas inhibition of ILK led to a dramatic coalescence and disorganization of stress fibers (Fig. 5f).

ILK Stimulates Stellate Cell Activation—Important features of stellate cell activation include enhanced fibrogenesis (expression of type I collagen and/or other matrix proteins) as well as expression of the cytoskeletal protein smooth muscle α-actin, a well accepted marker of stellate cell activation (19). To test the functional effects of ILK in stellate cell possibility, we first overexpressed ILK in isolated quiescent stellate cells. When ILK was overexpressed, we saw remarkable stimulation of type I collagen mRNA production, whereas abrogation of ILK expression led to a reduction in collagen I mRNA expression (Fig. 5g). Consistent with the effects of ILK on PI 3-kinase, as above, wortmannin inhibited the fibrogenic effect of ILK in stellate cells (Fig. 5g). Additionally, ILK overexpression led to dramatic up-regulation of smooth muscle α-actin, and as above, is a well accepted marker of stellate cell activation (Fig. 5h).

Blocking of the PI 3-kinase system inhibited the effect of ILK on smooth muscle α-actin (Fig. 5h).

ILK Modifies the Wound-healing Response to Injury in Vivo—Given our ability to manipulate ILK levels with the constructs

Constructs were not only active but also specific, because control sequences had no effect on ILK expression (Fig. 4a).

Functional Effects of ILK—An important phenotype for mesenchymal cells, such as stellate cells, during wound healing is cell adhesion. To assess cell adhesion, activated stellate cells were infected with Ad-GFP, Ad-ILK, or Ad-shILK (or were not infected (control)). Forty-eight hours after infection, cell adhesion was assessed (Fig. 5a). We found that overexpression of ILK led to enhanced cell adhesion, whereas ILK knockdown inhibited cell adhesion (Fig. 5a). Importantly, exposure of cells to wortmannin abrogated the effect of overexpression of ILK, consistent with a downstream effect of ILK on the PI 3-kinase pathway. To verify the role of PI 3-kinase in this system, we verified that activation of PI 3-kinase led to Akt phosphorylation (data not shown).

ILK Modulates hepatic fibrogenesis in vivo. Common bile ligation was performed as described under “Experimental Procedures.” During surgery, adenovirus (Ad-ILK, Ad-Sh-RNA, (shILK) or Ad-GFP, as described under “Experimental Procedures” was injected via the inferior vena cava (a concentration of 1 × 10¹⁰ plaque-forming units/kg in 500 μl of PBS). Ten days later, as shown in a–d, livers were harvested and subjected to sirius red staining as described under “Experimental Procedures.” Representative liver sections are shown from rats undergoing sham BDL (a), BDL (b), or those additionally exposed to Ad-Sh-RNA (c) or Ad-ILK (d). The scale bar shown in the lower right of d represents 50 microns. e, histomorphometric analysis was performed on sirius red-stained liver sections as described under “Experimental Procedures” (n = 10 fields/liver, and 10 livers/group; *, p < 0.05 for BDL compared with their respective sham conditions; #, p < 0.05 for Ad-ILK/BDL or Ad-Sh-RNA/BDL compared with Ad-GFP/BDL). f, hydroxyproline content was measured in the livers of specific groups as described under “Experimental Procedures” (n = 10; *, p < 0.05 for BDL compared with their respective sham conditions; #, p < 0.05 for Ad-ILK/BDL or Ad-Sh-RNA/BDL compared with Ad-GFP/BDL).
ILK and Liver Fibrosis

FIGURE 7. ILK regulates smooth muscle α-actin, collagen I α1, fibronectin, and TGF-β expression in vivo. Liver injury and introduction of adenoviral vectors was as described in the legend to Fig. 6. Ten days after surgery, livers were harvested, total RNA was extracted, and real time PCR was performed as described under “Experimental Procedures” to detect collagen I α1 mRNA (a), smooth muscle α-actin (Sm α-actin) (b), TGF-β mRNA (c), and extra domain A (EDA) fibronectin (d) (n = 10; * p < 0.05 compared with the respective sham condition; # p < 0.05 for Ad-ILK/BDL or Ad-ShRNA/BDL compared with Ad-GFP/BDL).

highlighted in Fig. 4, we set out to determine whether ILK might play a role in in vivo wound healing, using the liver as a model. We administered relevant constructs to control or injured (BDL) rats and assessed typical wound-healing phenotypes. We found that overexpression of ILK during the wounding response led to markedly enhanced fibrogenesis (Fig. 6). Notably, we were able to verify quantitatively significant increases in ILK expression in whole liver extracts from these specific livers (data not shown); additionally, livers from these rats were palpably more firm and rigid than the controls. In contrast, inhibition of ILK led to reduced fibrogenesis (Fig. 6). Morphometric analysis of large numbers of liver sections revealed that ILK overexpression during the wounding response led to a dramatic increase in collagen, whereas inhibition of ILK led to a reduction in collagen (Fig. 6e). Consistent with the morphometric data, the hydroxyproline content of whole livers was elevated after ILK overexpression and reduced after ILK knockdown (Fig. 6f). We further assessed other fibrogenic fingerprints in vivo, including the expression of smooth muscle α-actin, type I collagen, TGF-β, and extra domain A fibronectin mRNA. These other fibrogenic markers were increased after ILK overexpression and reduced after ILK inhibition (Fig. 7). Smooth muscle α-actin also varied after ILK manipulation in vivo, consistent with the in vitro data shown in stellate cells in Fig. 5. Because smooth muscle α-actin is expressed by activated stellate cells, this finding is consistent with an effect of ILK on stellate cell activation.

DISCUSSION

The major finding of this work is that ILK appears to be important in eliciting a fibrogenic phenotype in hepatic stellate cells. We found that, in isolated cells, ILK overexpression stimulated type I collagen expression and, in vivo, overexpression of ILK in the whole liver led to an exaggerated wounding phenotype, data that together strongly support a role for ILK in fibrogenesis. This notable finding, although somewhat unexpected, is not necessarily surprising. For example, it is well appreciated that cell matrix interactions are important in fibrogenesis and wound healing (20–23). Thus, the finding that a cell matrix-signaling partner such as ILK plays a role in this process is predictable. Indeed, our data further emphasize the importance of the extracellular matrix and of molecules (i.e. ILK) that transduce its signals.

An important question surrounds the mechanism by which ILK stimulates collagen synthesis and fibrogenesis in stellate cells. We speculate there to be one of several mechanisms at work. First, it is possible that ILK signals directly to downstream cascades important in extracellular matrix synthesis. For example, it is well established that stimulation of ILK after exposure of cells to extracellular matrix or even soluble factors affect Akt and other effectors (i.e. inhibition of GSK-3, etc.). The PI3-kinase/Akt pathway, in particular, appears to be linked to collagen gene expression (24). It is noteworthy that we were able to clearly demonstrate that the PI 3-kinase is important in mediating the effects of ILK in stellate cells (8, 25, 26), demonstrating an effect of ILK on the cytoskeleton (20–23). Thus, the finding that a cell matrix-signaling partner such as ILK plays a role in this process is predictable. Indeed, our data further emphasize the importance of the extracellular matrix and of molecules (i.e. ILK) that transduce its signals.

Second, ILK could signal in an indirect manner. Our data suggest that together strongly support a role for ILK in fibrogenesis. This notable finding, although somewhat unexpected, is not necessarily surprising. For example, it is well appreciated that cell matrix interactions are important in fibrogenesis and wound healing (20–23). Thus, the finding that a cell matrix-signaling partner such as ILK plays a role in this process is predictable. Indeed, our data further emphasize the importance of the extracellular matrix and of molecules (i.e. ILK) that transduce its signals.

An important question surrounds the mechanism by which ILK stimulates collagen synthesis and fibrogenesis in stellate cells. We speculate there to be one of several mechanisms at work. First, it is possible that ILK signals directly to downstream cascades important in extracellular matrix synthesis. For example, it is well established that stimulation of ILK after exposure of cells to extracellular matrix or even soluble factors affect Akt and other effectors (i.e. inhibition of GSK-3, etc.). The PI3-kinase/Akt pathway, in particular, appears to be linked to collagen gene expression (24). It is noteworthy that we were able to clearly demonstrate that the PI 3-kinase is important in mediating the effects of ILK in stellate cells (8, 25, 26). However, we cannot exclude other pathways. For example, an additional potential signaling pathway might include Rho, with perhaps an effect on the cytoskeleton. Second, ILK could signal in an indirect manner. Our data showing that ILK has important effects on the stellate cell cytoskeleton (Fig. 5, c–f) are consistent with previous work demonstrating an effect of ILK on the cytoskeleton (8, 25, 26), raising the possibility that signaling to the cytoskeleton ultimately activates extracellular matrix synthesis pathways. Finally, ILK appears to have adapter capabilities (4, 5, 27, 28) that could facilitate fibrogenesis. For example, ILK interacts with PINCH and paxillin and is capable of forming a complex that co-localizes with integrins in focal adhesions. Thus, ILK could act as an
adapter connecting integrins with components of important extracellular matrix synthesis signaling pathways.

It was noteworthy that ILK overexpression appeared to prominently enhance the fibrogenic phenotype after a fibrogenic stimulus. That is to say that, after BDL, there was an exaggerated fibrogenic response. We speculate that one reason for this marked potentiation of the wounding phenotype has to do with known cell matrix biology. It is well appreciated that interactions between cells and their surrounding environment influence cell function. For example, after liver injury, the extracellular matrix changes rapidly, and, moreover, it specifically contributes to activation of hepatic stellate cells (1, 29). Matrix produced after liver injury in vivo, which is rich in extra domain A fibronectin, is a particularly strong stimulus of stellate cell activation (30). Thus, we speculate that the overexpression of ILK in stellate cells already surrounded by a (permissive) matrix facilitated stellate cell activation and the fibrogenic response.

ILK is an intracellular serine/threonine protein kinase that interacts with the cytoplasmic domains of integrins and numerous cytoskeleton-associated proteins (5–7). ILK is also involved in the regulation of a number of integrin-mediated processes, including cell adhesion, cell shape changes, and gene expression, all of which are important in the wound-healing response (31–33). These data are consistent with emerging data linking ILK to a wounding phenotype. For example, ILK appeared to facilitate TGF-β-mediated epithelial to mesenchymal transition after ureteral obstruction, a process thought to be important in renal fibrosis (34). Our data in an isolated cell model additionally raise the possibility that ILK could be important in other systems in which mesenchymal cells that resemble stellate cells (i.e. dermal myofibroblasts, mesangial cells, pancreatic stellate cells, etc.) are important in the fibrogenic response. For example, in preliminary experiments, we found that ILK is expressed in isolated mesangial cells and is up-regulated after growth in culture, similar to the situation in hepatic stellate cells.

Consistent with previous data that emphasize the functional effects of ILK on a variety of cellular processes, we found that overexpression of ILK specifically led to significant changes in stellate cell adhesion and cell motility. This raises the possibility that a functional effect such as cell motility, adhesion, or spreading could be linked to collagen synthesis. Available data suggest that stellate cells are highly motile (18) and that their motility could be important in the evolution of the wound-healing response. We speculate that, if enhanced cell motility is linked with a wound healing phenotype, this may proceed via ILK-induced cytoskeletal changes.

We used adenovirus to transduce gene products in stellate cells in vitro. Although we did not directly study the transduction efficiency of our adenoviral constructs in vivo in this study, previous data demonstrated that, when adenovirus is injected systemically, both in normal liver and in injured liver, adenovirus leads to high level transduction of multiple cell types, including stellate cells (35), and moreover, we verified expression of ILK after Ad-ILK infection of the liver.

Another important finding of this work was that ILK appears to be regulated after liver injury. We found that molecules important in the wounding process, such as TGF-β (36) and ET-1 (37), stimulated the expression of ILK. An important question is whether compounds such as ET-1 or TGF-β act through ILK or act perhaps more directly. Available data suggest that ET-1 or TGF-β may have direct effects on matrix synthesis in stellate cells (38, 39), but whether intermediates such as ILK are involved has remained largely unexplored. A final point is that ILK is likely to be regulated in injury characterized by a wound-healing response; this finding will likely be reproduced in wounding models in a variety of tissues.

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ILK and Liver Fibrosis

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