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Life Sciences 78 (2006) 1794 – 1800
Life Sciences
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Up-regulation of integrin-linked kinase activity in rat mesangioproliferative glomerulonephritis

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Received 10 June 2005; accepted 11 August 2005

Abstract

This study investigated whether integrin-linked kinase (ILK) is involved in the pathogenesis of chronic glomerulonephritis (GN) by analyzing the expression and activity of glomerular ILK in a chronic rat model of mesangioproliferative GN. Double immunostaining of kidneys obtained at different time points with glomerular cell-specific markers revealed that ILK was primarily expressed by glomerular epithelial cells, and weakly by mesangial cells (MCs) and endothelial cells in control rats, but dramatically increased in a typical mesangial pattern at days 21 and 28 of GN. Semiquantitative assessment indicated that the level of glomerular ILK expression closely parallels the level of accumulation of glomerular extracellular matrix (ECM) as well as fibronectin (FN). Immunoprecipitation and kinase activity assays using isolated nephritic glomeruli indicated a striking increase of ILK activity on days 21 and 28 of GN. Further, cultured rat MCs overexpressing kinase-deficient ILK diminished FN assembly and collagen matrix remodeling as compared with control transfectants. The results showed that glomerular ILK expression and activity are markedly increased in an experimental model of chronic GN. Increased activity of ILK in MCs may contribute to the development of chronic mesangial alterations leading to glomerular scarring.

Keywords: Integrin-linked kinase; Extracellular matrix; Glomerulonephritis; Progressive renal disease; Mesangial cell

Introduction

Sustained glomerular hypercellularity and abnormal mesangial extracellular matrix (ECM) remodeling are the main pathological features of chronic glomerulonephritis (GN) leading to glomerular dysfunction and scarring (sclerosis) (Border and Noble, 1994). They are further characterized by the increase of mesangial cell (MC) number and accumulation of fibronectin, laminin and collagen type IV (collagen IV), and the neoexpression of collagen I and III in diseased glomeruli (Kagami et al., 2004). Therefore, elucidating the molecular and cellular mechanisms responsible for persistent pathological mesangial remodeling is essential for understanding the pathogenesis of progressive GN.

Accumulating evidence indicates that β1 integrin receptors (β1 integrins), mainly mediating cell adhesion to ECM components, contribute to tissue repair and fibrosis through modulating cell behaviors such as cell proliferation, survival, apoptosis, migration and ECM assembly (Danen and Yamada, 2001; Kagami and Kondo, 2004). These biological potentials of β1 integrins are mediated through the interaction of the cytoplasmic domain of integrins with associated signaling molecules, including nonreceptor tyrosine kinases such as focal adhesion kinase (FAK), integrin-linked kinase (ILK), adaptor proteins such as Grb-2, p130Cas, and structural proteins such as paxillin and talin. Recently, along with others, we reported that α1β1 integrin, a collagen and laminin receptor, plays a critical role in ECM remodeling and cell proliferation in diseased kidneys (Cosgrove et al., 2000; Cook et al., 2002; Kagami et al., 2002). Thus, targeting integrin-mediated

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doi:10.1016/j.lfs.2005.08.037

signaling pathways that regulate glomerular cell growth and ECM assembly may lead to a new strategy for prevention of the progression of glomerular diseases.

ILK is an intracellular serine/threonine kinase that interacts with β1 integrin and has been implicated in the regulation of cytoskeletal organization, cell proliferation, survival, apoptosis, and assembly of ECM such as fibronectin (FN) (Hannigan et al., 1996; Wu and Dedhar, 2001). There are some reports on the roles of ILK in renal diseases. Kretzler et al. (2001) reported that ILK activity in glomerular podocytes played a crucial role in podocyte detachment leading to glomerular dysfunction. Furthermore, Guo et al. (2001) reported that ILK expression increased in the expanded mesangium in patients with diabetic nephropathy. Additionally, they found that the PINCH-ILK-CH-ILKBP complex regulated proliferation of MC and deposition of FN, suggesting that ILK was involved in mesangial ECM accumulation and cell proliferation in diseased kidneys (Guo and Wu, 2002). To further investigate whether ILK is involved in the pathogenesis of chronic GN, expression and activity of glomerular ILK was investigated in a chronic model of rat mesangioproliferative GN induced by injection of anti-Thy-1 monoclonal antibody (mAb) 1-22-3 to uninephrectomized rats (Nakamura et al., 1999). Additionally, the potential role of ILK activity in ECM assembly was studied in cultured MCs.

Materials and methods

Experimental model and design

Progressive anti-Thy-1 nephritis was induced in male Sprague–Dawley rats ($n=30$) (SLC, Shizuoka, Japan), weighing 150 to 160 g at the start of the experiment, by a right-sided uninephrectomy. One week later, rats received a single intravenous (tail vain) injection of 2 mg of monoclonal antibody (mAb) 1-22-3 (Nakamura et al., 1999). Controls ($n=6$) received vehicle only. Six rats at each time point were sacrificed on days 3, 7, 14, 21 and 28 after injection of mAb 1-22-3. Six rats were sacrificed before injection of mAb1-22-3 as a baseline control (0 h). In addition, five rats (Nx group) were injected with phosphate-buffered saline (PBS) 1 week after uninephrectomy, and sacrificed on day 28 after injection.

Urinary protein excretion

Urine was collected from rats in a metabolic cage for 24 h, at 1 week after surgery, and on days 3, 7, 14, 21 and 28 after mAb 1-22-3 or PBS injection. The amount of urinary protein excretion was measured by the Bradford method (Bio-Rad, Oakland, CA, USA).

Histology and immunohistochemistry

The right kidney of each rat was processed and examined by light, immunofluorescence (IF) as previously described (Kagami et al., 2002). Frozen sections (3 μm) were fixed in acetone. Then, they were incubated with goat anti-ILK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h, and subsequently with fluorescein isothiocyanate (FITC)-coupled donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, Pennsylvania). In double staining experiments, sections were further incubated with either mouse monoclonal anti-α-SM actin antibody (1A4; Sigma Chemical Co., St. Louis, Missouri), mouse monoclonal anti-Thy-1 antibody (Taiho Pharmaceutica Co., Tokushima), mouse monoclonal synaptopodin antibody (G1D4; Progen Biotechnik, Heidelberg, Germany) or mouse monoclonal anti-rat RECA-1 antibody (HIS 52; Serotec Ltd., Kindlington, UK), followed by an appropriate tetramethylrhodamine isothiocyanate (TRITC)-coupled donkey anti-mouse antibody (Jackson Immuno-Research Laboratories, Inc.). Negative controls included the omission of either of the primary antibodies for which no double staining was noted.

For evaluating the level of glomerular staining with either anti-ILK antibody or anti-FN antibody (Chemicon International, Temecula, CA), semiquantitative analysis was performed and ranked as follows: 0, diffuse, very weak or absent mesangial staining; 1+, diffuse, weak mesangial staining with 1% to 25% of focally increased mesangial staining; 2+, 25% to 50% of glomerular tuft demonstrating strong mesangial staining; 3+, 50% to 75% of glomerular tuft demonstrating strong mesangial staining; and 4+, 75% or more of glomerular tuft stained strongly. For each kidney section, 30 glomeruli were selected at random and were evaluated by a blinded observer and the mean value per section was calculated.

For semiquantitative evaluation of mesangial matrix accumulation, all 3-μm PAS-stained sections were coded and read by a blinded observer. At least 30 glomeruli were selected at random, the degree of glomerular matrix expansion was determined using a published method (Kagami et al., 2002). The percentage of each glomerulus occupied by mesangial matrix was estimated and assigned a code beginning with 1=0–25%, 2=25–50%, 3=50–75%, 4=75–100%.

ILK activity

Isolated glomeruli were lysed in an ice-cold lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) in the presence of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Precleared cell lysates (100 μg) were incubated with mouse monoclonal anti-ILK antibody (65.1.9) (Upstate Biotechnology, Inc., Lake Placid, NY) and protein G beads overnight at 4 °C. Immune complexes were washed twice with lysis buffer followed by two washes with kinase buffer (Cell Signaling Technology, Inc.). Washed pellets were incubated with kinase buffer supplemented with 200 μM ATP and 250 ng of the commercially available Akt/PKBa (inactivate) (Upstate Biotechnology, Inc.) for 60 min at 30 °C. The kinase reaction was terminated by addition of SDS sample buffer and the supernatants were boiled for 5 min at 100 °C and resolved by SDS/PAGE (12.5% gels). Membranes were probed with Phospho-Akt (ser473) antibody (Cell Signaling Technology, Inc.), followed by incubation with an HRP-conjugated anti-rabbit antibody. Immunoreactive protein was detected with an

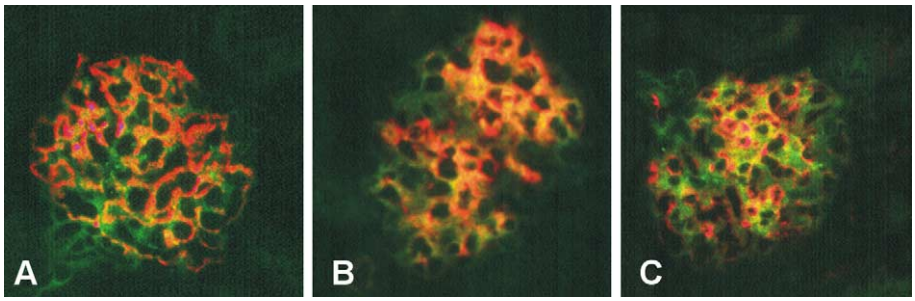

Fig. 1. Double immunostaining of ILK (FITC in green) and glomerular cell-specific antigens (TRITC in red) in normal rat glomeruli. Kidney sections stained with goat anti-ILK antibody (A, B, C) and FITC-labeled anti-goat antibody were double stained with either mouse monoclonal anti-synaptopodin antibody (podocyte marker) (A), mouse monoclonal anti-Thy-1 antigen antibody (mesangial cell marker) (B) or mouse monoclonal anti-rat RECA-1 antibody (endothelial cell marker) (C), followed by TRITC-labeled anti-mouse antibody. Yellow staining indicates areas of simultaneous expression of ILK and synaptopodin, anti-Thy-1 antigen, or RECA-1 (Magnification ×200) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

Rat mesangial cell culture and transfectants

Cultured rat mesangial cells (MCs) were established from intact glomeruli of Sprague–Dawley rats as previously described (Kagami et al., 2004). Stable transfectants expressing flag-tagged kinase-deficient ILK (K220M) (Yamaji et al., 2001), were generated as follows. Using a modified calcium phosphate co-precipitation method, MCs (5×10^5) were doubly transfected with 5 μg of pME18s flag-ILK (K220M) and 0.5 μg of pRc/CMV2 (Invitrogen, San Diego, CA), which introduces a neomycin phosphotransferase gene. Stable transfectants were selected in the presence of neomycin analogue Geneticin (G418; Gibco BRL, Orland Island, NY) (0.75 mg/ml) and subcloned by limiting dilution to generate single-cell subclones. Control clones, Mocks, were also established by double-transfection with pME18s flag and pRc/CMV2. Protein expression of flag-ILK was confirmed with Western blotting analysis using mouse monoclonal anti-flag antibody (M2) (Sigma Chemical Co.).

Immunofluorescent staining of MCs

Rat MC transfectants were cultured on wells of Lab-Tek 8-chamber culture slides (Nunc, Inc.) in DMEM medium supplemented with 10% fetal bovine serum (Gibco BRL) and G418. Cells were fixed with acetone, permeabilized with 0.05% Triton X-100 in PBS containing 1 mg/ml bovine serum albumin, and then stained with mouse monoclonal anti-ILK antibody (65.1.9) (Upstate Biotechnology, Inc.) and rabbit anti-FN antibody.

Collagen gel contraction assay

Collagen gel assays were performed to examine the ability of cells to reorganize and contract three-dimensional collagen I gels. Quiescent transfectants were harvested, suspended at a concentration of 5×10^5 cells/ml, and mixed with collagen solution (59% 1.25× RPMI 1640, 1% 0.2 N NaOH, 40% rat tail collagen I (3.75 mg/ml)). Collagen/cell suspension was incubated in 12-well plate (1 ml/well) (Costar, Cambridge, MA) at 37 °C to polymerize the collagen. At selected time points, the diameter of the hydrated gels was measured by use of an inverted microscope.

Statistical analysis

Data were collected from three independent experiments of duplicate or triplicate wells. Data are presented as mean ± S.D. A value of $p < 0.05$ was considered significant (Student's *t*-test).

Results

Proteinuria and light microscopic findings

In uninephrectomized rats (nephritic rats) that received mAb 1-22-3, urinary protein excretion was increased on day 3 (39.4 ± 11.8 mg/24 h), on day 7 (86.9 ± 21.2 mg/24 h) and then transiently decreased on day 14 (58.2 ± 8.6 mg/24 h). However, it rose again to 99.8 ± 29.7 mg/24 h on day 21, and was 124.5 ± 31.0 mg/24 h on day 28. No abnormal proteinuria was detected in the uninephrectomized rats without an injection of 1-22-3 during the experimental period. Glomerular alterations were examined on days 3, 7, 14 and 28 after mAb 1-22-3 injection. Light microscopy showed almost all glomeruli had mesangiolytic changes on day 3, followed by MC proliferation accompanied by mesangial matrix expansion on day 7. At this stage of GN, severe lesions such as mesangial balloons and aneurysms were still observed in many glomeruli. Thereafter, massive accumulation of mesangial ECM and MC proliferation were the prominent features of nephritic glomeruli on day 28. No abnormal morphological alteration was observed in the uninephrectomized rats without injection of mAb 1-22-3. The results obtained from this study performed on Sprague–Dawley rats are similar with a previous study reporting that mesangial alteration induced by mAb 1-22-3 injection into uninephrectomized Wistar rats was prolonged (Nakamura et al., 1999).

Expression of ILK and FN in normal and nephritic rat glomeruli

Double immunostaining of kidney with glomerular cell-specific markers revealed that ILK was primary expressed by

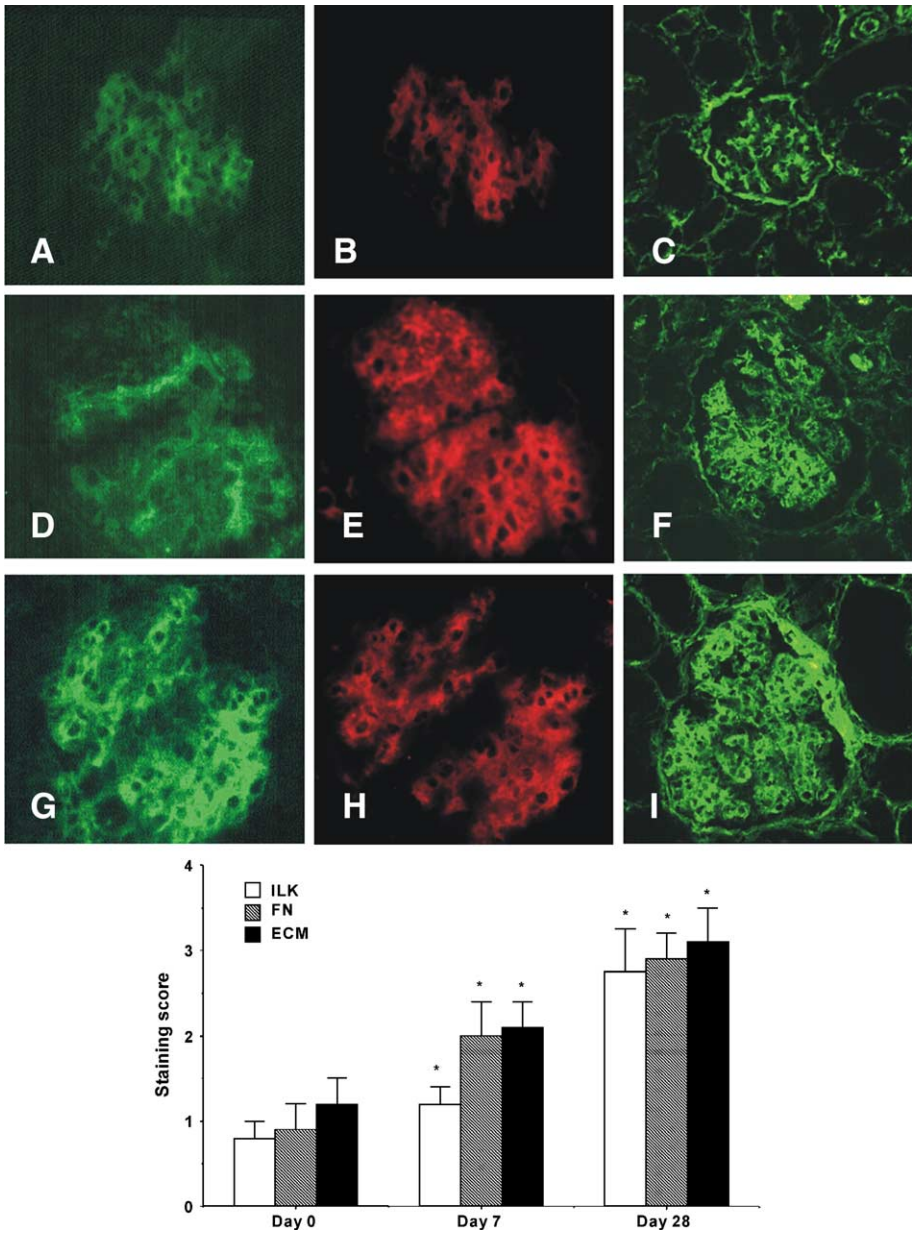

Fig. 2. Glomerular expression of ILK, FN (FITC in green) and Thy-1 antigen (TRITC in red) in rats with chronic anti-Thy-1 GN. Kidney sections from days 0 (A, B, C), 7 (D, E, F) and 28 (G, H, I) nephritic rats were double stained with anti-ILK antibody (A, D, G) and anti-Thy-1 antigen monoclonal antibody (B, E, H). In addition, sections from days 0, 7 and 28 nephritic rats were stained with anti-FN antibody (C, F, I) and subsequently with FITC-labeled anti-rabbit antibody (Magnification ×200). (J) Semiquantitative assessment of ILK and FN and extracellular matrix (ECM) accumulation in days 0, 7 and 28 nephritic rats. Data are shown as means ± S.D. (*$p < 0.01$ vs. rats at day 0) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

glomerular epithelial cells, and to a lesser extent by MCs and faintly by endothelial cells in control rats (Fig. 1). However, on day 7, ILK staining was slightly enhanced in expanded mesangium and was dramatically increased in a typical mesangial pattern on day 28 of GN (Fig. 2). Since in several cell culture studies (Wu et al., 1998; Guo et al., 2001), overexpression of ILK was reported to promote FN matrix assembly, correlation of ILK expression and FN accumulation in diseased glomeruli was examined. The results showed a coordinated increase of ILK and FN expression in glomerular mesangium (Fig. 2). Furthermore, semiquantitative assessment indicated that the level of glomerular ILK expression closely paralleled the levels of accumulation of glomerular FN as well as ECM during the course of chronic anti-Thy-1 glomerular disease ($p < 0.01$) (Fig. 2J).

ILK activity and protein expression in nephritic glomeruli

Glomerular ILK activity during the course of anti-Thy-1 GN was examined using isolated control and nephritic glomeruli. To estimate ILK activity, we employed an in vitro kinase assay using inactivated Akt/PBK protein substrate because Akt (serin 473) has been shown to be a target of ILK activation (Wu and Dedhar, 2001). As shown in Fig. 3, there

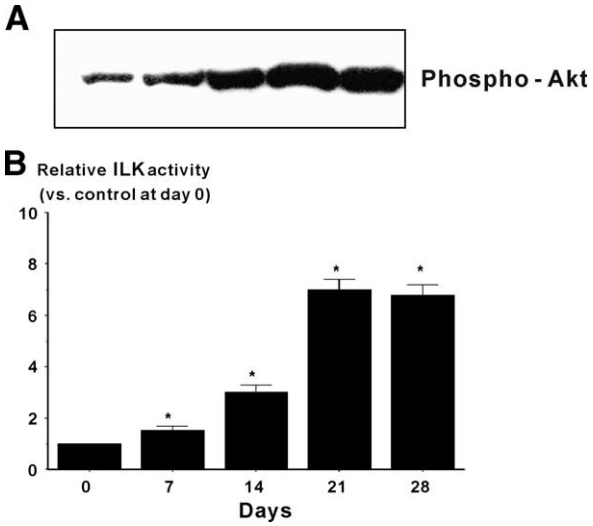

Fig. 3. Time course of glomerular ILK activity in rats with chronic anti-Thy-1 GN. (A) The glomerular cell extracts (200 μg) at indicated time points were subjected to immune complex kinase assay. ILK activity was assayed by detecting phosphorylated Akt (Phospho-Akt) by Western blot analysis. (B) Kinase assay was quantitated using LKB UltroScan XL apparatus, and fold activation is indicated as relative ILK activity. Data are shown as means±S.D. (*$p<0.01$ vs. rats at day 0).

was a gradual increase of ILK activity, which showed a marked increase on day 21 (7-fold of control) and lasted at least until day 28 of GN. This finding was closely associated with the time course of glomerular ILK expression by immunofluorescence examination.

Inhibition of ILK activity reduced ECM assembly by MCs

To test whether ILK activity influences MC behavior involved in the progression of GN, we established cultured MC lines that were transfected with either kinase-deficient ILK or mock DNA. As shown in Fig. 4, neomycin-resistant, two stable kinase-deficient-transfectants (B4, B5), and two mock-transfectants (F1, F3) showed different profiles of protein expression and kinase activity. Kinase-deficient ILK

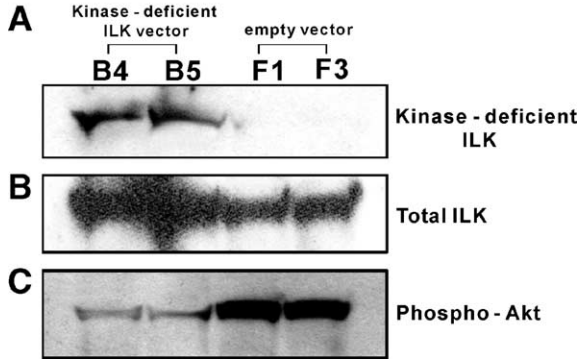

Fig. 4. Western blot analysis of either kinase-deficient ILK vector or empty vector-transfected MCs. (A) A monoclonal anti-flag antibody was used to detect the presence of enforced expression of kinase-deficient ILK. (B) Anti-ILK antibody was used to detect endogenous ILK and exogenous ILK proteins (total ILK) in transfectants. (C) ILK activity in transfectants was detected as a phosphorylated Akt (Phospho-Akt) by using immune complex kinase assay.

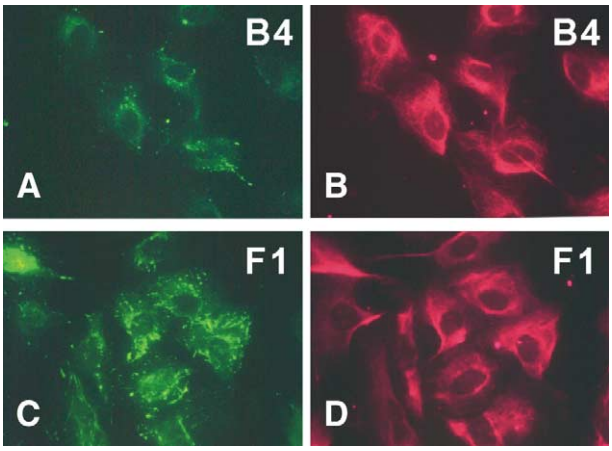

Fig. 5. FN matrix assembly by either kinase-deficient ILK vector- or empty vector-transfected MCs. FN matrix assembly was analyzed by immunofluorescent staining of cell monolayers. Either kinase-deficient ILK vector-transfected MC clones (B4) or empty vector-transfected MC clones (F1) were fixed with acetone, permeabilized with 0.05% Triton X-100 in PBS, and then double stained with polyclonal rabbit anti-FN antibody (A, C) and mouse monoclonal anti-ILK antibody (B, D) (Magnification ×200).

transfectants, which were positive for flag protein, showed an increased ILK protein but decreased ILK activity compared with mock-transfectants. The functional significance of ILK activity in ECM assembly by MCs, an important cellular process involved in the pathogenesis of glomerulosclerosis, was evaluated by cell culture studies. As shown in Fig. 5, kinase-deficient ILK-transfectants showed diminished FN fibril formation compared with mock-transfectants. Furthermore, gel contraction assay indicated that kinase-deficient ILK-transfectants had less ability to reorganize collagen matrix compared with mock-transfectants (Fig. 6). Thus, these results suggested that ILK activity was critically involved in mesangial ECM assembly.

Discussion

The present results provide the first evidence that glomerular ILK expression and activity are increased in disease progression in an experimental model of rat mesangioproliferative GN. Increased ILK expression in expanded mesangium of nephritic glomeruli was associated with abnormal ECM accumulation. Further, the molecular effect of overexpressing a dominant-negative, kinase-deficient form of ILK on MCs indicates that the

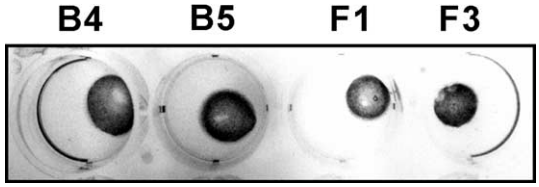

Fig. 6. Collagen matrix remodeling activity by either kinase-deficient ILK vector- or empty vector-transfected MCs. Ability of MCs to remodel collagen matrix was examined by collagen gel contraction assay. Either kinase-deficient ILK-transfected MC clones (B4, B5) or empty vector-transfected MC clones (F1, F3) were mixed with collagen type I solution and then incubated in a 12-well plate for 24 h. A representative experiment is shown.

increase of ILK activity in nephritic glomeruli seems likely to up-regulate ECM assembly by MCs in GN. These results suggested that β1 integrin cytoplasmic domain-binding protein, ILK is an important signaling molecule necessary for MC-related ECM deposition, which is a prominent biological feature of chronic progressive GN.

Increasing evidence indicates that β1 integrins contribute to the development and progression of glomerular injury (Kagami and Kondo, 2004). Immunohistological study shows that the expression levels of glomerular β1 integrins (α1β1, α5β1 integrins) parallel the severity of glomerular ECM deposition and cellularity in rat Thy-1 GN and human GN such as IgA nephropathy, lupus GN and diabetic nephropathy (Kagami et al., 1993; Kuhara et al., 1997; Jin et al., 1996). Administration of function-blocking anti-β1 integrin antibody into rats in two experimental models of GN revealed that α1β1 integrin was involved in the development of GN characterized by MC proliferation and ECM accumulation (Kagami et al., 2002; Cook et al., 2002). Genetic knock-out studies have also implied a critical role for β1 integrins in glomerular ECM assembly (Cosgrove et al., 2000). Thus, ILK may possibly mediate glomerular function of β1 integrins in GN.

Integrin-mediated signals play a crucial role in the modulation of cell phenotype such as cell proliferation, migration, apoptosis (survival) and ECM assembly (Danen and Yamada, 2001; Kagami and Kondo, 2004). Especially, recent evidence indicates that FN assembly is regulated by α5β1 integrin-mediated signaling (Wierzbicka-Patynowski and Schwarzbauer, 2003). In vitro data reveals that down stream signal molecules following α5β1 integrin-mediated cell adhesion include FAK, Src and PI-3K, which play a role in FN assembly (Ilic et al., 1995; Wierzbicka-Patynowski and Schwarzbauer, 2002). The present study using MC clones overexpressing kinase-deficient ILK showed that ILK was a critical signaling molecule in MC-induced FN fibril formation. Similar results have been obtained using the same approach, i.e., ILK activity was shown to be involved in FN assembly in cultured rat intestinal epithelial cells (IEC-18) and human kidney tubular cells (Wu et al., 1998; Li et al., 2003). Recently, Guo and Wu (2002) reported that ILK formed a tertiary complex with PINCH and CH-ILKBP in rat MCs, which were co-clustered at fibrillary adhesion sites involved in FN matrix deposition. They demonstrated that inhibition of PINCH-ILK-CH-ILKBP complex formation significantly reduced FN matrix deposition, suggesting that the PINCH-ILK-CH-ILKBP complex also critically participates in the regulation of mesangial FN matrix deposition in GN.

Interestingly, collagen gel contraction assay, in an in vitro model of collagen matrix remodeling and scar formation (Kagami and Kondo, 2004), by MC clones overexpressing kinase-deficient ILK showed that ILK activity is also involved in the collagen matrix remodeling process by MCs. In general, the gel contraction process involves several cell behaviors, including cell adhesion to collagen via integrins, rearrangement of the surrounding collagen fibrils, and cell contraction and migration within collagen lattices. We recently demonstrated that the extracellular signal-regulated kinase (ERK) pathway was critical to the α1β1 integrin-dependent MC migration necessary for collagen matrix remodeling (Kagami et al., 2001). Based on other experimental results using either kinase-deficient ILK expressing cells or RNA interference-induced ILK depleted cells, ILK appears to negatively influence cell behaviors such as migration and contraction and thereby may inhibit gel contraction (Kim et al., 2004; Vouret-Craviari et al., 2004). Additional studies are needed to explore the cellular mechanisms responsible for the decreased ability of kinase-deficient ILK expressing MCs in collagen matrix remodeling. Taken together, ILK activity seems to be important for MC-related glomerular ECM remodeling activity. To investigate how ILK links to the other signaling molecules described above, a detailed analysis of the ILK signaling network involved in MC-induced ECM assembly needs to be performed.

ILK has been implicated in the development of human renal diseases. Guo et al. (2001) found that glomerular mesangial ILK expression was dramatically increased in patients with diabetic nephropathy; the increases associated with diffuse mesangial expansion. They showed that the increase of ILK expression in MCs could be induced by hyperglycemia, a causal factor of diabetic glomerulosclerosis. Furthermore, Kretzler et al. (2001) using a differential display screening identified ILK as a candidate downstream effector in proteinuria in children with congenital nephrotic syndrome of the Finish type. They showed that ILK expression and activity were involved in podocyte functions such as adhesion and shape changes that could lead to podocyte detachment from the basement membrane and severe proteinuria. Emerging evidence suggests that renal tubular epithelial cells can undergo epithelial–messenchymal transformation (EMT) to become ECM-producing fibroblasts under pathological conditions (Li et al., 2003; Liu, 2004). Li et al. (2003) reported that ILK was a critical mediator for TGF-β1-induced renal tubular EMT and likely played a crucial role in the development of chronic renal fibrosis. In summary, ILK likely represents a key regulator of the cellular behavior involved in the progression of renal diseases.

Conclusions

The present study showed that glomerular ILK expression and activity were markedly increased in an experimental model of chronic GN. ILK activity appeared linked to ECM remodeling activity by MCs. We propose that controlled regulation of glomerular ILK activity could provide the basis for an effective therapeutic method for inhibition of pathological mesangial ECM remodeling in disease progression.

Acknowledgments

This work was supported by grants from the Japanese Ministry of Welfare (14570748, 16591035). We thank Naomi Okamoto for her excellent technical assistance. Parts of this work were published in abstract form at the 34th Annual Meeting of the American Society of Nephrology, October 30–November 4, 2002.

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