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Integrin-linked kinase is involved in matrix-induced hepatocyte differentiation

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Abstract

Hepatocytes have restricted proliferative capacity in culture and when cultured without matrix, lose the hepatocyte-specific gene expression and characteristic cellular micro-architecture. Overlay of matrix-preparations on de-differentiated hepatocytes restores differentiation. Integrin-linked kinase (ILK) is a cell-matrix-adhesion protein crucial in fundamental processes such as differentiation and survival. In this study, we investigated the role of ILK, and its binding partners PINCH, α -parvin, and Mig-2 in matrix-induced hepatocyte differentiation. We report here that ILK is present in the liver and localizes at cell-matrix adhesions of cultured hepatocytes. We also show that ILK, PINCH, α -parvin, and Mig-2 expression level is dramatically reduced in the re-differentiated hepatocytes. Interestingly, hepatocytes lacking ILK undergo matrix-induced differentiation but their differentiation is incomplete, as judged by monitoring cell morphology and production of albumin. Our results show that ILK and cell-matrix adhesion proteins play an important role in the process of matrix-induced hepatocyte differentiation.

Keywords

ILK; PINCH; Parvin; Mig-2; Hepatocytes; Differentiation; Matrix

Hepatocytes, the parenchymal cells of the liver, have restricted proliferative capacity when maintained in culture and they de-differentiate over time losing both their hepatocyte-specific gene expression patterns and their characteristic cellular micro-architecture. Interestingly, addition of hydrated complex matrix preparations (Matrigel: a matrix extract from Engelbreth-Holm-Swarm (EHS) mouse sarcomas or Type I Collagen Gels) [1] over de-differentiated hepatocytes restores differentiation within 3 days [2,3]. This highlights the importance of matrix for hepatocyte differentiation and normal function.

Signals from the extracellular matrix (ECM) are transmitted to the cells through integrins. Integrins in turn associate with a number of integrin-proximal proteins and, through those interactions, signals are transduced to actin cytoskeleton or the nucleus, thus enabling the cell to respond properly to environmental changes and signals [4].

Integrin-linked kinase (ILK) is an important component of cell-matrix adhesions that has been shown to be critically involved in many fundamental cellular processes such as survival and differentiation [5–10]. Furthermore, ILK over-expression has been associated with anchorage-independent growth, suppression of anoikis, and oncogenic transformation in general [11–14].

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ILK can function as an adaptor protein in the cell and is capable of mediating several protein–protein interactions with other cell-ECM adhesion proteins. For instance, ILK interacts with the $\beta 1$ and $\beta 3$ subunits of integrin [12], thus linking the ECM signaling to the intracellular compartment of the cell. Moreover, ILK has been shown to bind to PINCH and α -parvin, which are also focal adhesion proteins, forming a stable ternary complex at the cell-ECM adhesions sites [15,16]. In fact, the PINCH–ILK–parvin complex is pre-formed and recruited to the cell-ECM adhesion sites as a complex [17], and it has been implicated in several crucial cellular processes. Furthermore, each one of the components of the complex has been shown to be crucial for cell survival [18,19], and cell spreading. ILK is also capable of binding to another focal adhesion protein termed Mitogen-inducible gene 2 (Mig-2) [20], which is also important for the regulation of cell shape and spreading [21].

Finally, it is worth noting that ILK, as well as PINCH, α -parvin, and Mig-2 are all widely expressed in the human tissues and well conserved among different species [16,20,22], suggesting an essential role in many different organisms and processes.

In the present study, we investigated the role of ILK as well as its binding partners PINCH, α -parvin, and Mig-2 in hepatocyte differentiation induced by matrix. Primary hepatocytes were isolated from the rat liver by collagenase perfusion and cultured for 8 days in collagen-coated plates. As shown previously [2], hepatocytes de-differentiated over time in culture and addition of Matrigel restored differentiation within 3 days. This model culture system for studying hepatocyte differentiation was previously used to determine the expression and distribution of focal adhesion (integrins, focal adhesion kinase, paxillin) and cell adhesion molecules (E-cadherin) [3], as well as the regulation of the β -catenin pathway [23].

In the present study, we show that ILK is present in the rat liver and localizes at the cell-ECM adhesion sites of primary rat hepatocytes in culture. Interestingly, the expression level of ILK in whole cell lysates of re-differentiated hepatocytes following Matrigel overlay is reduced, and this reduction is even more striking when we examine the Triton X-100-soluble cellular fraction during re-differentiation, suggesting that ILK is involved in cellular control of hepatocyte differentiation induced by matrix. Furthermore, we show that the expression level of PINCH, α -parvin, and Mig-2 is also dramatically reduced during the re-differentiation process. Interestingly, hepatocytes isolated from conditional ILK-knock-out mice which lack ILK in the liver underwent differentiation in response to matrix but the differentiation was impaired compared to that of the control ILK-containing cells, as judged by cell morphology and albumin production. Our results suggest that ILK and its binding partners play an important role in the matrix-induced hepatocyte differentiation, although further studies are needed to decipher the mechanism governing this complex cellular process.

Materials and methods

Antibodies and reagents

Matrigel was purchased from BD Bioscience (BD Bioscience, Bedford, MA). Plates were coated with 10% Vitrogen (Cohesion, Palo Alto, CA), and were air-dried under a hood and ultraviolet light overnight.

The following primary antibodies were used in this study: mouse monoclonal (mAb) anti-ILK antibody (clone 69), rabbit polyclonal anti-PINCH, mAb anti- α -parvin (clone 3B5), mAb anti-Mig-2 antibody as described previously [18,19,21], sheep anti-rat albumin (Immunology Consultants Laboratory, Newberg, OR), and anti- β -actin mAb (Chemicon, Temecula, CA). All secondary Abs were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Hepatocyte isolation from rat liver and Matrigel overlay

Primary rat hepatocytes were isolated from male Fischer 344 rat livers by a calcium two step collagenase perfusion technique, as described previously [2]. Freshly isolated rat hepatocytes were plated at a density of 400,000 cells/ml, on collagen-coated 6-well plates, in MEM medium (Sigma, St. Louis, MO) supplemented with 500 ng/ml insulin and 50 mg/ml gentamycin. Three to 4 h after plating, the medium was replaced with hepatocyte growth medium (HGM) containing 40 ng/ml epidermal growth factor (EGF) and 20 ng/ml hepatocyte growth factor (HGF) as described previously [2]. The rat hepatocytes were kept in culture for a total of 15 days and the HGM medium was changed every two days.

Rat hepatocytes in half of the plates were overlaid with diluted Matrigel on the eighth day following the liver perfusion (day 0 corresponds to the day of the perfusion), and cultured for one week, while the rest of the hepatocytes were maintained in normal culture conditions without Matrigel treatment. Matrigel was diluted 1:3 with HGM, and the medium was changed in all cultures every 2 days.

Generation of ILK-knock-out animals in the liver

ILK floxed animals were generated as described previously [10] and donated by Drs. René St. Arnaud (Shriners Hospital and McGill University, Montréal) and Shoukat Dedhar (British Columbia Cancer Agency and Vancouver Hospital, Jack Bell Research Center, Vancouver), and mated with AFP-enhancer-albumin-promoter-Cre-recombinase-expressing mice which were kindly provided by Dr. Klaus Kaestner (University of Pennsylvania). The off-spring were genotyped as described previously [10] and the ILK-floxed/floxed Cre-positive mice were considered to be ILK-knock-out (ILK-KO) while their Cre-negative siblings were used as controls (Gkretsi V. and Michalopoulos G.K., manuscript under review).

Hepatocyte isolation from mouse liver and Matrigel overlay

Primary mouse hepatocytes were isolated from the livers of ILK-KO and their normal siblings by a calcium two step collagenase perfusion technique, as described above. Freshly isolated hepatocytes were plated at a density of 300,000 cells/ml, on collagen-coated 6-well plates, in MEM medium (Sigma, St. Louis, MO) supplemented with 500 ng/ml insulin and 50 mg/ml gentamycin. Three to four hours after plating, the medium was replaced with HGM similar to the one used in rat cultures [2] but with the following two differences; instead of DMEM-based HGM that is used in rat cultures, we used MEM-based HGM in the mouse cultures and the dexamethasone added was one tenth of the one used in the rat cultures (10^{-8} M). HGF and EGF were added in the same concentrations as described above [2]. The mouse hepatocytes were kept in culture for a total of 11 days and the MEM-based-HGM medium was changed every two days.

Matrigel was diluted in the MEM-based-HGM and added to the mouse hepatocyte cultures on day 8 as described above for the rat. Cells were examined daily and pictures were taken. Cells were harvested on day 10 (3 days following Matrigel overlay).

Immunofluorescent staining

Immunofluorescent staining was performed as described previously. Briefly, cells were plated on collagen-coated coverslips, fixed with 4% paraformaldehyde and stained with the anti-ILK mouse monoclonal antibody (clone 69) or anti-Mig-2 mouse monoclonal antibody. A rhodamine-red conjugated goat anti-mouse antibody was used as a secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Immunohistochemistry

Immunohistochemical staining for ILK and Mig-2 was performed on formalin-fixed paraffin embedded livers, following standard immunohistochemical procedures as described previously [24].

Protein isolation and immunoblotting analysis

Total cell lysates were isolated from Matrigel-treated primary rat hepatocytes at different time points following the overlay of Matrigel (days 10 and 15) and at the same time points lysates were isolated from untreated cultures. Whole cell lysates were generated using 1% sodium dodecyl sulfate (SDS) in RIPA buffer (20 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 0.25% sodium deoxycholate (DOC), 0.6–2 µg/ml aprotinin, 10 µM leupeptin, and 1 µM pepstatin). The Triton X-100-soluble fractions were isolated by lysing the cells for 30 min on ice in RIPA buffer without SDS, followed by centrifugation at 14,000 rpm for 15 min, and collection of the supernatant. Protein concentrations of all lysates were determined using the bicinchoninic acid protein assay reagents (BCA method) (Pierce Chemical Co., Rockford, IL). Lysates were run on 10% reducing SDS–polyacrylamide gels. Actin was used as a loading control.

Results and discussion

ILK and Mig-2 are both present in the liver and they localize at the cell-ECM adhesions of primary rat hepatocytes in culture

We first set out to test the expression and localization of ILK and Mig-2 in the liver. To do that, we performed immunohistochemical staining on formalin-fixed paraffin-embedded liver sections. As shown in Fig. 1, both ILK and Mig-2 are present in the liver (Fig. 1A–C). In fact, Mig-2 appears to be more concentrated in the periportal and centri-lobular regions (Fig. 1C).

Both ILK and Mig-2 have been shown to be present in the cell-ECM adhesions sites of multiple cell types [21,25]. To test whether the localization is the same in hepatocytes, we performed cell immunostaining of primary rat hepatocytes cultured on collagen. Indeed, both ILK (Fig. 1E) and Mig-2 (Fig. 1F) are abundant in primary cultured rat hepatocytes, forming nice and strong focal adhesions. A picture of the cells stained with just the secondary antibody (Fig. 1D) serves as negative control for the cell immunostaining.

Differentiation of hepatocytes is restored following addition of Matrigel

Intrigued by the finding that ILK is abundant at the cell-ECM adhesion sites of primary hepatocytes in culture, we sought to find whether its total expression level changes during hepatocyte differentiation induced by matrix. For that purpose, primary rat hepatocytes were cultured for eight days in collagen-coated plates using the chemically defined hepatocyte growth medium (HGM), which allows serum-free clonal expansion of primary hepatocytes in the presence of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [2]. During that time the albumin gene expression was dramatically reduced showing that the cells started to de-differentiate (Fig. 2A, compare albumin in lane 1 with lanes 2 and 3) as described previously [2]. On the eighth day, Matrigel was added on half of the plates with the de-differentiated hepatocytes and re-differentiation occurred as shown by the increased albumin expression (Fig. 2A, compare lanes 2 and 3 to lane 4) as well as the characteristic hepatocyte morphology (data not shown) as described previously [2]. Albumin was used as a hepatocyte-specific differentiation marker and the results were consistent with previous studies done in the same model system of matrix-induced hepatocyte differentiation [2,3], demonstrating that Matrigel overlay indeed restores hepatocyte differentiation.

In order to investigate the role of ILK in this process, we analyzed the total protein level of ILK in de-differentiated and re-differentiated hepatocytes. As shown in Fig. 2A, ILK total level was significantly reduced following Matrigel overlay in primary cultured hepatocytes (Fig. 2A, compare lanes 2 and 3 to lane 4) almost returning to the levels on day 1, while albumin level has also been restored to the initial levels (Fig. 2A, compare lanes 1 and 4). This result suggests that ILK is involved in the cellular control of hepatocyte differentiation induced by matrix, and that a relatively low level of ILK is characteristic of differentiated hepatocytes (Fig. 2A, lanes 1 and 4).

The total protein expression level of ILK, PINCH, and α -parvin is greatly reduced during the matrix-induced hepatocyte differentiation, while the level of Mig-2 is not dramatically affected

To gain further knowledge on the role of ILK in the matrix-induced hepatocyte differentiation, we analyzed the total expression level of the ILK-binding proteins, PINCH, α -parvin, and Mig-2. As shown in Fig. 2B, the total levels of all three components of the PINCH–ILK–parvin complex were reduced following addition of Matrigel over de-differentiated hepatocytes (Fig. 2B, compare lanes 2 and 3, for ILK, PINCH, and α -parvin). Furthermore, the total expression level of Mig-2, another ILK-binding protein, was also reduced but not as dramatically (Fig. 2B, compare lanes 2 and 3 for Mig-2).

In a study by Kim et al. [3] using the same model culture differentiation system, it was shown that the expression level of paxillin and focal adhesion kinase (FAK), two other focal adhesion proteins, is increased in the Matrigel-treated hepatocytes. Thus, the data of the present study and the study by Kim et al., suggest that during the matrix-induced hepatocyte differentiation, there is an interesting re-distribution of the different focal adhesion molecules, and some are increased while others are decreased as a response to Matrigel overlay and matrix in general. Therefore, understanding the regulation or the mechanism behind this interesting process would provide new insights into hepatocyte biology and thus facilitate hepatocyte-oriented therapeutic approaches.

The expression levels of ILK, PINCH, α -parvin, and Mig-2 are dramatically reduced in the Triton X-100-soluble cellular fraction during hepatocyte differentiation

To shed more light upon the mechanism regulating this complex process of matrix-induced hepatocyte differentiation, we examined the intracellular distribution of ILK, PINCH, and α -parvin during the re-differentiation process. Triton X-100-soluble cellular fractions, which contain the cytosol, were isolated from de-differentiated or re-differentiated primary rat hepatocytes (at least 3 days following the addition of Matrigel). As shown in Fig. 3, ILK, PINCH, α -parvin, and Mig-2 levels were dramatically reduced in the re-differentiated hepatocytes following Matrigel overlay (Fig. 3A–D, respectively). These findings show that all members of the PINCH–ILK–parvin complex and the ILK-binding protein Mig-2 are dramatically down-regulated from the cytosol during the matrix-induced hepatocyte differentiation, suggesting a biological relevant role of the PINCH–ILK–parvin complex in hepatocyte biology and the process of differentiation.

Consistent with the above, a similar reduction in ILK in the Triton X-100-soluble cellular fraction was also observed during the process of myogenic differentiation [8], indicating that ILK plays an important role in differentiated cells and corroborating the finding of the present study.

Regarding the cellular distribution of Mig-2, the total expression of which was not as dramatically reduced following Matrigel overlay (Fig. 2B), we found that it was significantly reduced in the Triton X-100-soluble or cytosolic fraction (Fig. 3D), while it was increased in

the Triton X-100-insoluble fraction (data not shown). This explains why there was no significant change in the total expression level of Mig-2 (Fig. 2).

It is worth noting that all proteins examined in this study, namely ILK, PINCH, α -parvin, and Mig-2 have reduced expression levels in the Triton X-100-soluble cellular fractions which correspond to the cytosol. In contrast, other focal adhesion proteins such as FAK, and paxillin [3], are elevated in those fractions, indicating the complexity of the mechanism regulating the matrix-induced hepatocyte differentiation.

Hepatocytes lacking ILK do re-differentiate following Matrigel overlay but their re-differentiation is incomplete

In order to further investigate the importance of ILK for the process of matrix-induced hepatocyte differentiation, we used the loxP/Cre system and mouse hepatocytes that lacked ILK. Thus, we mated ILK-floxed animals [10] with a strain of transgenic animals that express Cre-recombinase under the control of a hepatocyte-specific promoter; Alpha Fetoprotein (AFP) enhancer-albumin promoter. The AFP-enhancer-albumin-promoter is activated after liver specification and becomes stronger after birth along with albumin expression. Studies from our lab have shown that ILK is indeed removed specifically from the hepatocytes of these animals while it is still present in the non-parenchymal cells of the liver and that these ILK-KO livers are characterized by a dramatic decrease in differentiation markers (Gkretsi V. and Michalopoulos G.K., manuscript under review).

Thus, hepatocytes isolated from ILK-KOs and Cre-negative animals were put in culture and Matrigel was added on day 8 in accordance with the experiments done in rat hepatocytes. As shown in Fig. 4 (lower panel), hepatocytes lacking ILK were smaller and looked less differentiated than the control hepatocytes at Day 10 (Fig. 4, lower panel, compare C and D to A and B). Addition of Matrigel induced increase in albumin expression in the wild-type cells (Fig. 4, upper panel, compare albumin for lanes 1 and 2) and a slight increase was observed in the ILK-KO cells (Fig. 4 upper panel, compare albumin for lanes 3 and 4). Interestingly, comparison between the albumin expression level of the WT and the ILK-KO-hepatocytes (Fig. 4 upper panel) shows a dramatic downregulation in the KO-cells, indicating a significant role played by ILK for the maintenance of hepatocyte differentiation.

In the present study, we have shown that the redistribution of components of the cell-ECM adhesion molecules is an important element of restoring differentiation in Matrigel-treated hepatocyte cultures and that ILK is important regulator of that process. Although the molecular mechanisms governing the hepatocyte differentiation process are far from being deciphered, the present work provides new insight that could enhance our understanding of the whole process, and ultimately enable us to apply this information in the field of cell transplantation of hepatocytes in critically ill liver patients or other hepatocyte-based therapies.

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Fig. 1.

Immunohistochemical localization of ILK and Mig-2 in the liver and localization of ILK and Mig-2 at the cell-ECM adhesions of primary rat hepatocytes. (A) Formalin-fixed-paraffin embedded liver sections from normal rat liver were stained with anti-ILK antibody (20× magnification). (B) Formalin-fixed-paraffin embedded liver sections from normal rat liver were stained with anti-ILK antibody (40× magnification). (C) Formalin-fixed-paraffin embedded liver sections from normal liver were stained with anti-Mig-2 antibody (10× magnification). (D–F) Primary rat hepatocytes stained with (D) the secondary antibody only (as a negative control), (E) the anti-ILK antibody, and (F) the anti-Mig-2 antibody.

Fig. 2.

Hepatocytes re-differentiate in culture following addition of Matrigel (MTG), and the total expression level of ILK, PINCH, α -parvin, and Mig-2 is reduced during that process. (A) Western blot of total cell lysates from hepatocytes harvested on Days 1, 8, and 10 following isolation from the liver. Matrigel (MTG) was added on Day 8. Western blot using an anti-rat-albumin antibody (upper panel) or the anti-ILK antibody (bottom panel). Note that the same membrane was first probed with ILK and then re-probed without stripping with the anti-rat albumin antibody. (B) Western blot analyses of whole cell lysates from primary rat hepatocytes treated with matrigel (MTG) on Day 8 post isolation, using antibodies against ILK, PINCH, α -parvin, and Mig-2. β -Actin serves as a loading control.

Fig. 3.

Expression level of ILK, PINCH, α -parvin, and Mig-2 in the Triton X-100-soluble cellular fraction during the matrix-induced hepatocyte differentiation. Triton X-100-soluble fractions from de-differentiated and re-differentiated hepatocytes 10 and 15 days after the addition of Matrigel (MTG) were subject to Western blot analysis for (A) ILK, (B) PINCH, (C) α -parvin, and (D) Mig-2. β -Actin is used as a loading control.

Fig. 4.

Hepatocytes lacking ILK re-differentiate following Matrigel (MTG) overlay but the differentiation is incomplete. Upper panel: Western blot analysis of total cell lysates from hepatocytes isolated from WT and ILK-KO mouse livers on Day 10 with and without the addition of MTG. The same blot was used for albumin and β -actin without stripping. Lower panel: Cell morphology of hepatocytes on Day 10: (A) WT hepatocytes cultured without MTG, (B) WT hepatocytes after the addition of MTG, (C) ILK-KO hepatocytes cultured without MTG, and (D) ILK-KO hepatocytes after the addition of MTG.