Ezrin is required for epithelial-mesenchymal transition induced by TGF-β1 in A549 cells

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Abstract. Epithelial mesenchymal transition (EMT) has been shown to play a role in cellular differentiation during development and tumor invasion. However, the precise molecular mechanisms of EMT are not fully elucidated. Previous studies suggested that the mechanism underlying the possible involvement of ezrin in EMT process might be different from that of moesin, another ERM protein. In our study, we examined the role of ezrin in actin filament reorganization and cell metastasis during TGF-β1-induced alveolar EMT. Suppressing ezrin expression limited morphological changes and actin filament remodeling, decreased cell migration and invasion during EMT. Immunofluorescence experiments indicated that EMT characteristics in lung cancer cells are associated to differential ezrin subcellular localization. We also found that podocalyxin interacted with ezrin after TGF-β1 induction. Therefore, ezrin is an important regulator of the EMT process, and its function might possibly be mediated by the ezrin-podocalyxin interaction during TGF-β1-induced alveolar EMT. Our finding provides important new insights into the mechanisms of action of the ERM proteins in the TGF-β1-induced alveolar EMT.

Introduction

Epithelial mesenchymal transition (EMT) not only plays crucial roles in embryonic development or tissue repair, but is also involved in fibrotic diseases and cancer progression (1-3). During EMT, cells undergo profound phenotypic changes including the loss of cell-cell adhesion, the loss of cell polarity, the reorganization of cytoskeleton, and the acquisition of migratory and invasive properties (4,5). A network of transcriptional regulators is involved in the control of EMT, which is coupled to posttranscriptional and posttranslational modifications that amplify the initial signals (6). Several transcription factors have been shown to be involved in this process, such as the Snail family of zinc-finger transcription factors and the basic helix-loop-helix factors Twist (7,8). These transcription factors downregulate epithelial markers (E-cadherin) and upregulate mesenchymal markers (fibronectin, vimentin), which induce EMT and consequently promote the development of metastatic properties (9,10). Many extracellular matrix components and cytokines, including Wnt, hepatocyte growth factor, epidermal growth factor (EGF) and transforming growth factor-β (TGF-β), can elicit EMT. Among them, TGF-β is a powerful inducer of EMT (11). Depending on the specific cellular context, different signaling pathways during TGF-β1-induced EMT can be activated and contribute to establish an organizing center that in turn controls morphogenetic movement and specification (12).

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic progressive fibrosing interstitial pneumonia of unknown cause that is limited to the lungs (13). A major factor in IPF pathogenesis is thought to be an aberrant activation of alveolar epithelial cells (AECs). AECs can transformed into (myo) fibroblasts, which secrete an excessive amount of collagen to form fibers, impairing organ function (14). There is increasing evidence that EMT is involved in these processes during pulmonary fibrogenesis and TGF-β is a major inducer of EMT in the lungs (2). Previous studies indicated the alveolar epithelial cells (AEC) underwent EMT during the pulmonary fibrosis induced by TGF-β, and TGF-β1 induced A549 AEC to undergo EMT via Smad2 activation (15,16). These results suggest that AEC serve as a source of fibroblasts in lung fibrosis and highlight the potentially critical role of EMT in the induction of fibrosis in the lung. Core of this process is cytoskeleton reorganization (5). After induction by proinflammatory cytokines, actin filament architecture changes from cortical actin to stress fibers. However, the precise mechanism underlying these structural rearrangements remains unknown.

Ezrin, radixin and moesin, known as the ERM proteins, are a group of membrane-cytoskeleton linkers, which are closely associated with actin cytoskeleton remodeling (17,18). Two groups have shown the role of ERM proteins in the actin filaments rearrangement during EMT (19,20). ERM proteins may be involved in regulating alveolar structure and lung homeostasis. Moreover, dysregulation of the ERM-RAGE...
complex might be an important step in rearrangement of the actin cytoskeleton during proinflammatory cytokine-induced EMT of human alveolar epithelial cells. Recent study showed that increased moesin expression promotes EMT by regulating actin filament remodeling, but ezrin expression decreased in NMuMG cells or remained unchanged in A549 cells during TGF-β-induced EMT, suggesting that the regulating mechanism of EMT by ezrin may be different from that by moesin if it does play a role in this process (21).

Podocalyxin (PODXL), as a member of the CD34 family, is a type I transmembrane glycoprotein, playing an important role in regulating cell adhesion and cell morphology (22). It has a number of interacting partners, including the actin binding protein ezrin, the adhesion molecule L-selectin and Na+/H+ exchanger regulatory factor (NHERF) (23,24). PODXL could increase migration and invasion, MMP expression, and activation of MAPK and PI3K activity in MCF7 and PC3 cells through interaction with ezrin (25). Moreover, PODXL is markedly increased and required for TGF-β induced EMT of A549 cells (26). During this process, PODXL interacts with collagen type I, which may control cell migration by regulating the dynamics of cell protrusion formation.

In our study, we examine the role of ezrin in actin filament reorganization and cell metastasis during TGF-β1-induced alveolar EMT. Our finding also revealed an association between ezrin and PODXL during EMT, suggesting ezrin-podocalyxin complex might be important in actin filament remodeling during TGF-β1-induced alveolar EMT.

Materials and methods

Antibodies. Monoclonal ezrin antibody, monoclonal or polyclonal podocalyxin antibodies and monoclonal vimentin antibody were obtained from Santa Cruz Biotechnology. Monoclonal E-cadherin antibody was purchased from BD (Becton-Dickinson Co.). Polyclonal GAPDH antibody was from Cell Signaling Technologies (Beverly, MA, USA). Monoclonal β-actin antibody was from Proteintech. Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 was from Invitrogen (Carlsbad, CA, USA).

Cell culture and treatment. Human bronchoalveolar carcinoma cell H358, human lung adenocarcinoma cell A549 and human lung adenocarcinoma cell H1299 were purchased from Thermo Scientific Dharmacon. The cells were transfected with ezrin siRNA or negative control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA interference. Sequences of ezrin siRNA were designed and synthesized by Thermo Scientific Dharmacon. Ezrin siRNA contains 4 individual siRNAs, Ezrin-siRNA-1: 5'-GCU CAA AGA UAA UGC UAU GTT-3' (sense) and 5'-CAU AGC AUU AUC UUU GAG CTT-3' (antisense). Ezrin-siRNA-2: 5'-GGA AUC AAC UAU UUC GAG ATT-3' (sense) and 5'-UCU CGA AUA AGU UGA UUC CTT-3' (antisense). Ezrin-siRNA-3: 5'-GCG CAA GGA GGA AGU UTT-3' (sense) and 5'-AAC UUC AUC CUC CUU GCG CTT-3' (antisense). Ezrin-siRNA-4: 5'-GGC CGG AGC UGU CUA GUG ATT-3' (sense) and 5'-UCA CUA GAC AGC UCC GCG CTT-3' (antisense). The negative control siRNA was also purchased from Thermo Scientific Dharmacon. The cells were transfected with ezrin siRNA or negative control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunofluorescent analysis. Cells were fixed with 4% paraformaldehyde for 30 min, permeated with 0.1% Triton X-100 for 10 min at room temperature (RT), followed by blocking in 10% normal goat serum for 1 h, and then incubated with the primary antibody at 4°C overnight. After washing, the slides were incubated with Alexa flour 488-conjugated secondary antibody, followed by nuclear counterstaining with DAPI for 10 min. F-actin was stained using rhodamine conjugated-phalloidin (Invitrogen). Cells were imaged using a 40X EC Plan Neofluar/1.30 oil immersion objective on an inverted laser-scanning confocal microscope (LSM510 META, Carl Zeiss) and images were captured using Zeiss software.

Coimmunoprecipitation. Cells were rinsed with PBS twice and lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) with 1X proteinase inhibitor cocktail (Roche), 1 mM sodium fluoride, and 1 mM sodium orthovanadate (Ameresco) for 30 min at 4°C. The lysates were clarified by centrifugation at 13200 rpm for 20 min. Protein concentrations were detected using the BCA protein assay kit. After pre-clear, 1.5 mg total protein was immunoprecipitated with the indicated primary antibody overnight, and incubated with 20 µl Protein A/G Plus-Agarose beads for 4 h at 4°C. The immunoprecipitates and the lysates were subjected to western blotting using the antibody indicated.

Western blotting. Cells were grown to confluence on culture dishes, and lysed in ice-cold cell lysis buffer. Lysates were centrifuged at 13200 rpm for 20 min at 4°C to obtain the proteins, and then protein concentration was determined by the BCA protein assay. The protein lysates were separated by SDS-PAGE and then transferred to PVDF membrane (Millipore). The membranes were blocked with 5% non-fat milk solution for 1 h at room temperature and incubated in primary antibody dissolved in block solution at 4°C overnight. The proteins were probed by antibody against ezrin, E-cadherin, vimentin or β-actin. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and visualized using ECL.

Wound-healing assay. Adhered cell monolayers were scratched with a 200-µl pipette tip and grown in DMEM medium with 10% FBS at 37°C with 5% CO2. Wound healing capacity was monitored by microscopy at 0, 12 and 24 h.

Cell invasion assay. After siRNA transfection, TGF-β1 induction was started 48 h before the assay. The inserts were precoated with 40 µl BD Matrigel and then cells (5x10^4) were seeded in the upper chamber and incubated for 12 h. The cells

Wetstern blotting. Cells were grown to confluence on culture dishes, and lysed in ice-cold cell lysis buffer. Lysates were centrifuged at 13200 rpm for 20 min at 4°C to obtain the proteins, and then protein concentration was determined by the BCA protein assay. The protein lysates were separated by SDS-PAGE and then transferred to PVDF membrane (Millipore). The membranes were blocked with 5% non-fat milk solution for 1 h at room temperature and incubated in primary antibody dissolved in block solution at 4°C overnight. The proteins were probed by antibody against ezrin, E-cadherin, vimentin or β-actin. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and visualized using ECL.
were fixed and stained with crystal violet. Migrated cells in 5 randomly chosen fields of each well were counted.

Results

Suppressing ezrin expression limits morphological changes and actin filament remodeling during EMT. The morphological and biochemical features of A549 cells after TGF-β1 treatment were examined to confirm that EMT properties were induced under our culture conditions. After TGF-β1 treatment, A549 cells underwent phenotypic changes including the acquisition of spindle shape, the reorganization of actin filaments from cortical thin bundles to thick parallel bundles or actin stress fibers. In addition, the loss of expression of the epithelial marker, E-cadherin, and an increase of expression of the mesenchymal marker, vimentin further confirmed that EMT properties were induced under our culture conditions (Fig. 1).

To examine the functional significance of ezrin during EMT, we chose to knock down ezrin expression by using siRNA. Two days following the transfection with ezrin siRNA, the level of ezrin expression was reduced by ~70% as compared with the cells transfected with control siRNA (Fig. 2A and B). Additionally, there was no significant difference in LDH release in ezrin siRNA transfected cells as compared with the control, suggesting ezrin siRNA has no cytotoxicity on the cells tested (Fig. 2C). After TGF-β1 treatment, control cells expressing negative control siRNA showed decreased expression of E-cadherin and increased expression of vimentin during EMT. Cells transiently transfected with ezrin siRNA exhibited increased expression of E-cadherin and decreased expression of vimentin in contrast to negative control siRNA cells (Fig. 3A).

Compared with negative control siRNA cells, ezrin siRNA cells treated with TGF-β1 had different morphology and actin filament remodeling. After TGF-β1 treatment, actin filaments in negative control siRNA cells changed from a cortical actin network to thick bundles, or actin stress fibers and the cells elongated and became spindle shape. However, cells transfected with ezrin siRNA had fewer and thinner actin filaments, accompanied by incomplete morphological transition as compared with control siRNA cells (Fig. 3B). These results indicate that ezrin is associated with morphological changes and actin filament remodeling during EMT.

Suppressing ezrin expression during EMT decreases cell migration and invasion. The acquisition of migratory and invasive properties is one of the phenotypic changes during EMT. To determine the relationship between ezrin and cell migratory and invasive abilities during EMT, we used ezrin siRNA to suppress ezrin expression and observed cell migration and invasion after TGF-β1 treatment. In a wound-healing assay, cells transfected with ezrin siRNA showed decreased wound healing at the indicated time as compared with control siRNA cells (Fig. 4A). Moreover, the invasive potential of cells transfected with ezrin siRNA decreased as compared with control siRNA cells (Fig. 4B and C). These results demon-
Ezrin is involved in TGF-β1-induced alveolar EMT

Differential ezrin localization is associated with EMT characteristics in lung cancer cells. To further assess the involvement of ezrin expression in TGF-β1-induced EMT, we observed the change of ezrin expression level in A549 cells treated with TGF-β. However, the result showed that the expression level of ezrin has no change in A549 cells following TGF-β treatment (Fig. 5A), which is consistent with the report from Haynes et al (21). Subsequently, we observed the change of ezrin expression level in different lung cancer cell lines (H358, A549 and H1299). Though these cell lines underwent EMT to different extent, there was no obvious difference in the levels of ezrin protein (Fig. 5B). However, we found that ezrin was differently distributed in these lung cell lines (Fig. 5C). In H358 cells, ezrin was localized at microvilli throughout the entire cell. In contrast, A549 cells showed that ezrin was distributed in the cytoplasm. In H1299 cells, ezrin was found in the cytoplasm, and a proportion of it was colocalized with F-actin. The localization of ezrin and its colocalization with F-actin were in accordance with the invasion ability of these cell lines, which is one of the EMT properties. Taken together, the results demonstrated that differential ezrin localization, rather than total ezrin protein levels, is associated with EMT characteristics in lung cell lines, suggesting ezrin may be involved in the mechanism of TGF-β1-induced EMT through the change of its distribution.

The association of ezrin and podocalyxin increases during EMT. A recent study found that PODXL is involved in the EMT process through regulating the loss of epithelial features and acquisition of a motile phenotype. PODXL expression was induced upon TGF-β1 treatment (26). We asked whether PODXL is associated with ezrin during TGF-β1-induced EMT, and then used coimmunoprecipitation to detect the interaction between PODXL and ezrin. Our result showed that PODXL interacted with ezrin during TGF-β1-induced EMT (Fig. 6). These results suggest that ezrin may be involved in regulating TGF-β1-induced EMT through interaction with PODXL.

Discussion

A number of investigations into the molecular events of TGF-β1-induced EMT have paved the way for the design of improved specific therapies. In the present study, we provide information regarding the role of ezrin in TGF-β1-induced EMT, which may be associated with its localization and target proteins. These observations broaden knowledge of the precise molecular mechanism mediating TGF-β1-induced EMT, which is important for developing strategies to inhibit or reverse EMT.

Ezrin, as a membrane-cytoskeleton linker, plays a pivotal role in tumor invasion and metastasis (27-29). Ezrin can regulate the assembly of cytoskeleton elements to promote cytoskeletal reorganization and phenotypic alternation in cells, and facilitate cell migration and invasion (30,31). Overexpression of ezrin has been shown to enhance metastatic potential in various types of tumors, while downregulation of ezrin reduced the expression of β-catenin but enhanced the expression of E-cadherin (32,33). It has been found that ERM proteins are involved in regulating cytokine-induced EMT of human alveolar epithelial cells (19,20). The expression of moesin increased, which promotes EMT by regulating actin filament remodeling. However, the expression of ezrin has no change during TGF-β1-induced EMT (21). The role of ezrin in TGF-β1-induced EMT remains unknown. In the present study,
Figure 3. Suppressing ezrin expression limits morphological changes and actin filament remodeling during EMT. (A) The changes in protein expression during EMT are shown. NC siRNA, and ezrin siRNA cells were treated without or with TGF-β1 for 48 h. Blots were probed with antibodies for E-cadherin, vimentin, and ezrin. β-actin from the same loading was used as control. (B) The changes in cellular morphology and actin filament during EMT were detected. NC siRNA and ezrin siRNA cells were treated with TGF-β1 for 24 or 48 h. Fixed cells were incubated with anti-ezrin mAb (green) and rhodamine conjugated-phalloidin (red), and then photographed using a confocal microscope.

Figure 4. Suppression of ezrin expression during EMT decreases cell migration and invasion. (A) Ezrin siRNA knockdown decreases cell migration. NC siRNA and ezrin siRNA cells were treated with TGF-β1 for 48 h and scratched with a 200-µl pipette tip. Images were acquired by phase contrast microscope at the indicated time. (B and C) The cells transfected with NC siRNA or ezrin siRNA were seeded in the inserts precoated with BD Matrigel and incubated for 12 h. The NC siRNA and ezrin siRNA cells were quantified after treated for 48 h with TGF-β1. Data shown are means ± SEM from three independent cell preparations. *p<0.05, significant compared with the control group.
we demonstrated that ezrin is associated with morphological changes and actin filament remodeling, and regulates cell migration and invasion in TGF-β1-induced EMT, suggesting ezrin plays an important role in TGF-β1-induced EMT of human alveolar epithelial cells and its involvement is independent on the expression level (Figs. 3 and 4).

Several studies indicated that the localization of ezrin, rather than its expression level, is correlated with its function (34-36). In breast cancer lines, the total ezrin protein level has no difference, but its localization is associated with dedifferentiation and adverse features in invasive breast tumors and cancer cell lines. In salivary acinar cells from Sjögren's syndrome, the structure and organization of microvilli are linked to the localization changes of ezrin. In the present study, we also found that differential ezrin localization, rather than total ezrin protein levels, is associated with EMT characteristics in lung cell lines (Fig. 5). Increasing number of studies have shown ezrin is concentrated in the microvilli of the cell surface playing a normal role under normal physiological condition or before cytokine stimulation, while it could translocate to the cytoplasm after cytokine stimulation (37). In the cytosol, ezrin exists in a monomeric form and is thought to be ‘inactive’, provoking dysfunction in ezrin-mediated cellular processes (38-40). Following the stimulation, ezrin is phosphorylated, binds to actin and membrane proteins, colocalizes with F-actin, and redistributes to membrane, which is associated with enhanced migration and invasion (41-44). Our observations were very close to these studies (Fig. 3B). Before TGF-β1 induction, ezrin mainly distributes in the microvilli
and the nucleus. After TGF-β1 treatment, ezrin translocates to the cytosol, and with the increase of stimulation time, it co-localizes with F-actin and redistributes to cell membrane. These results indicated that ezrin may be involved in the mechanism of TGF-β1-induced EMT through the change of its distribution.

Ezrin is known to link membrane proteins and actin cytoskeleton. Membrane proteins include hyaluronate receptor (CD44), sodium-hydrogen exchanger (NHE), and cell adhesion molecules (ICAM-1, -2 and -3) (45-47). After activated with cytokine, membrane proteins could transduce extracellular signals into intracellular signals through the interaction between their extracellular domain and ligands. Ezrin also unmasks membrane protein and F-actin binding sites. The N-terminal FERM domain of ezrin can bind to the cytosplastic tail of membrane proteins and its C-terminal domain interacts with F-actin, resulting in actin filament remodeling. Ezrin interacts with actin and cytoplasmic tail of membrane proteins and its C-terminal domain interacts with F-actin, resulting in actin filament remodeling.

References

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