Original Article
Integrin β4 in EMT: an implication of renal diseases

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Abstract: Renal fibrosis is a main cause of chronic renal failure. Epithelial-to-mesenchymal transition (EMT) markers play a role in renal fibrosis. Transforming growth factor-β1 (TGF-β1) has been shown to initiate and complete the whole EMT process. It is now well accepted that loss of E-cadherin, EMT marker α-SMA, and connective tissue growth factor (CTGF) expression are key events in the EMT process. We found that by stimulating human renal proximal tubular epithelial (HK-2) cells with TGF-β1, the expression of E-cadherin was down regulated and the expression of α-SMA and CTGF were up regulated in a dose dependent manner. In our present study we also found that integrin β4 and peroxisome proliferators-activated receptor-γ (PPAR-γ) play roles in EMT process, with TGF-β1 stimulation increasing integrin β4 expression in HK2 cells. Integrin β4 and PPARγ were detected in tubulointerstitial tissues, immunohistochemistry analysis showed enhanced expression of integrin β4 in early stage, with over-expression at later stage. In contrast, the expression of PPARγ showed little increased in early stage, but was dramatically decreased at later stage. This is consistent with TGF-β1 inducing EMT. Our immune-precipitation studies show that integrin β4 disassociation with PPARγ is present in E-cadherin signaling. It suggests that PPARγ has a role in EMT inhibition.

Keywords: Integrin β4, PPARγ, EMT, renal diseases

Introduction

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional factor that regulates cell proliferation, differentiation, apoptosis, adhesion and migration, and induces the production of extracellular matrix proteins (ECM) [1]. Physiological levels of TGF-β1 are thought to be essential for normal development, tissue repair and maintenance of organ function. TGF-β1 has in anti-inflammatory actions in glomerular cells through the inhibition of mitogenesis and cytokine responses, and the suppression of accumulation and function of infiltrating cells. The overexpression of TGF-β1 has been linked with pathological alterations characteristic of various kidney diseases. In human glomerular disease, TGF-β1 has been reported as a key molecule that contributes to glomerulosclerosis [2]. It has been found that the urinary levels of TGF-β1 are elevated in patients with various renal diseases, increasing the amounts of interstitial fibrosis and mesangial matrix found. It has also been known that upregulated TGF-β1 stimulates the production of matrix proteins, decreases the activity of ECM-degrading proteinases and upregulates the synthesis of proteinase inhibitors, leading to excessive matrix deposition [2]. This suggests that TGF-β1 initiates a variety of pathophysiological processes early during kidney injury, including tubular epithelial cell apoptosis, intrinsic cell dedifferentiation, ECM deposition, and the development of renal fibrosis [3]. Progressive renal damage causes glomerulosclerosis, tubulointerstitial fibrosis, infiltration of inflammatory mediators and the activation of α-SMA-positive myofibroblasts. This acts as the final common pathway characteristic of all kidney diseases leading to chronic renal failure [4, 5].

It has been reported that TGF-β1 can control expression of αv, β4, and β1 integrin subunits in different cell type [8-13]. TGF-β1 signaling can also directly activate β1-integrin and induce cross-talk between different integrin and growth factor receptors. This includes the activation of focal adhesion kinase (FAK)-dependent clustering of ErbB2 (HER2) and integrin α6, β1, and β4 through a pathway initiated by epithelium growth factor receptor-dependent phosphorylation.
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Table 1. Primers used for RT-PCR amplification in the present study*

<table>
<thead>
<tr>
<th>Target</th>
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<th>Annealing (°C)</th>
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<td>Integran β4</td>
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<td></td>
<td>5'-CCTGGCGATGCGAGCAGAAG-3'</td>
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<tr>
<td>α-SMA</td>
<td>5'-AATGTAGAATGCTTGGG-3'</td>
<td>80</td>
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<td></td>
<td>5'-ACTAGTGAACTGTCAGAC-3'</td>
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<td>GAPDH</td>
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<td></td>
<td>5'-ACAAAGTGGTCGTTGAGGCA-3'</td>
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*E-cad: E-cadherin; Integran β4: Integrin Beta4; α-SMA: α-Smooth muscle actin; CTGF: Connective Tissue Growth Factor; GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase.

Epithelial-mesenchymal transition (EMT) is a process of epithelial cells losing their phenotypic markers and characteristics and acquiring the phenotypic features of mesenchymal cells. EMT is defined by four key events: (i) loss of epithelial cell adhesion molecules such as E-cadherin, (ii) α-Smooth Muscle Actin (α-SMA) and connective tissue growth factor (CTGF) expression, (iii) enhanced cell migration and invasion of the interstitium [6, 7]. It is clear that the well-described phenomenon of epithelial mesenchymal transition (EMT) plays a pivotal role in embryonic development, wound healing, tissue regeneration, and organ fibrosis and cancer progression.

We have previously found that TGF-β1 induced EMT by agonistic activation of PPARγ in human proximal tubule epithelial cells. Stimulation of human renal proximal tubular epithelial (HK-2) cells with TGF-β1 decreased E-cadherin and PPAR-γ expression level and increased connective tissue growth factor (CTGF) and α-smooth muscle actin (α-SMA) protein expression [14].

The mechanism of TGF-β1 induced EMT is not clear. We therefore specifically examined the role of E-cadherin, PPARγ and integrin β4 in EMT and renal fibrosis. We hypothesize that TGF-β1 induces EMT by agonistic activation of PPARγ mediated by E-cadherin and integrin β4.

Materials and methods

Cell culture and treatment

Immortalized human proximal tubule epithelial cells (HK-2) were obtained from Shenzhen Traditional Chinese Medicine Hospital (Shenzhen, China) and cultured as described previously [14]. Between passages 3 to 20, cells were plated in culture medium on 6-well plates on 1.5×10⁶ cells/well and allowed to grow for 48 hours. For cell treatment, starve cells 24 hours in medium without serum; and then cultured cells in DMEM containing TGF-β1 (R&D System, USA) in 0 ng/ml, 1 ng/ml, 3 ng/ml or 9 ng/ml for another 48 hours.

Human Embryonic Kidney 293 cells (HEK 293) were obtained from ATCC (American Type Culture Collection) and were grown in DMEM, supplemented with 10% calf serum or 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). The expression of E-cadherin in pcDNA3 vector was transfected into HEK 293 cells. After selection as described [16], stable cell line was obtained.

RT-PCR and gene expression analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was generated by reverse transcription 500 ng of immunosorbing total RNA in a reaction volume of 10 ul random hexamers as a priming agent with PrimeScript RT Master Mix (TAKARA, CHN). One microliters of cDNA was used as template in 20 ul PCR reaction. Quantitative real-time PCR was performed using Roche LightCycler 480 Real-Time PCR System with SYBR Premix Ex Taq (TAKARA, CHN). The number of PCR cycles used was determined to be within the linear range of the reactions. Sequences of primers and predicted PCR product sizes were shown.
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Figure 1. Measurement of mRNA level for E-cad, α-SMA and CTGF in absence or presence of TGF-β1 stimulation. The quantification of mRNA level of E-cad, α-SMA and CTGF by RT-PCR were shown by measurement in absence or presence of TGF-β1 (0, 1, 3 and 9 ng/ml) stimulation. Data were represented in triplicate experiments ± S.D.

Immuno-staining

Cells were washed twice in CMF-PBS and fixed for 12 hours in CMF-PBS + 4% paraformaldehyde at 4°C, followed by 3 times washes in PBS and blocking with PBS containing peroxidase inhibitor (MAIXIN, CHN) for 10 minutes at room temperature. Cells were incubated with 5 μg/ml mAb 3E1 (hybridoma facility, Memorial Sloan Kettering, New York, NY) diluted in blocking buffer for 12 hours at 4°C, washed four times with PBS, and incubated for 1 hour with anti-mouse IgG (2 μg/ml) conjugated with Alex488 (Invitrogen, USA) in blocking buffer, followed by five washes with PBS before mounting in Immumount (Shando). Images were acquired by using Leica DM4000 fluorescent microscope (Germany).

Histology and immunohistochemistry

The kidney tissues were obtained from 45 patients biopsy during 2013-2014 from the department of pathology, Peking University Shenzhen Hospital, Shenzhen, China. Kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin. All tissues were routinely stained with Hematoxilin & Eosin (H&E) and analyzed by a pathologist. According to the degree of tubulointerstitial lesion, kidney specimens were divided into 3 groups: mild lesion group (the tubulointerstitial lesion degree <25%), moderate lesion group (25%-50%), and severe lesion group (>50%). We used normal kidney portions as controls from two patients with localized clear cell carcinoma and from a patient underwent nephrectomy because of renal trauma.

For immunohistochemistry, slides were deparaffinized in xylene and dehydrated sequentially in ethanol. Antigen retrieval was performed in 0.01 M citrate buffer (pH6.0) for 30 minutes. Slides were quenched in peroxidase blocking reagent for another 30 minutes to block endogenous peroxidase activity. Slides were blocked with blocking buffer (PBS containing 10% goat serum), and then were incubated in diluted primary antibodies mouse anti-human integrin β4 (1:200) (Abcam) and PPARγ (Invitrogen) in PBS overnight at 4°C followed by incubation in secondary antibody goat anti-mouse Alexa Fluor 488 (1:250) (Invitrogen) at 37°C in the dark for 1 hour.
one hour. Between each step, slides were washed with PBS adequately. After washing, kidney tissues were double-stained with DAPI (LBP Medicine Science & Technology).

Figure 2. Immunofluorescence labelling of HK-2 cells for detecting expression of E-cadherin and α-SMA. Labelling HK-2 cells with mouse anti-human E-cadherin or mouse anti-human α-SMA followed by donkey anti-mouse Alexa Fluor 594 for α-SMA or goat anti-mouse Alexa Fluor 488 for E-cad in non-stimulation or stimulation with TGF-β1 (1, 3 and 9 ng/ml). Bar 20 μm.
Images were acquired by a Nikon Eclipse (Nikon, Aipidrag, Romania) microscope, equipped with a 5-megapixels CCD digital videocamera. Consecutive images, avoiding glomeruli, were recorded from the whole renal biopsy tissue at ×200 magnification.

Co-immunoprecipitation

Immunoprecipitations were carried in a manner similar as described [17]. Cells (8×10⁶) were washed once with washing buffer (50 mM Hepes, 50 mM NaCl and 10 mM EDTA, pH7.4), then lysed for 30 min on ice in 1% Triton X-100 containing a 1:1000 dilution of protease inhibitor mixture set III (Calbiochem, USA) in washing buffer. Cell debris was removed by centrifugation at 20,000× g for 30 min at 4°C. Cell lysates (1 mg of protein/ reaction determined by a BCA assay) were incubated at 4°C overnight with 10 µg/ml anti-PPARγ, Integrin β4, or E-cadherin antibodies or mouse IgG as negative control. Immune complexes were precipitated with 50 µl of GammaBind-Sepharose and washed with washing buffer. Samples were resolved by electrophoresis under reduced conditions on a 10% Laemmli gel [18], transferred to Immobilon P, and then probed with primary antibody followed by an alkaline phosphataseconjugated secondary antibody. Visualization of immunoreactive bands was performed using ECF reagent (GE Healthcare) and scanned on a Storm PhosphorImager (GE Healthcare).

Statistical analysis

Data were expressed as mean ± standard derivation. Unless stated, statistical significance was determined using Student’s t-test and statistical significance was achieved when the p value was <0.05.

Results

TGF-β1 decreased mRNA levels of E-cad and increased mRNA levels of α-SMA and CTGF in HK2 cells

In our previous study, we found that HK-2 cells were treated with TGF-β1 (3 ng/ml) 48 hours; the expression of EMT markers, α-SMA and CTGF were increased but E-cad decreased; loss of epithelial cell adhesion molecules such as E-cadherin was a key event of EMT. So the results was consistent the pattern of TGF-β1 induced EMT [14].

In present study, we treated HK-2 cells with TGF-β1 48 hours in different concentration (1, 3 or 9 ng/ml), E-cad, α-SMA and CTGF mRNA levels were measured by qRT-PCR assay. E-cadherin expression was dramatic decreased by TGF-β1treatment. TGF-β1 triggered significant increases of α-SMA and CTGF expression in dose-dependent manner (Figure 1). It was confirmed that TGF-β1 induced EMT in HK2 cells.
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TGF-β1 decreased protein expression level of E-cad and increased protein expression level of α-SMA in HK2 cells

To further understand TGF-β1 up-regulating α-SMA expression and down-regulating E-cadherin expression, we were observing the protein expression pattern in TGF-β1 stimulation by using immune fluorescence staining. It had been found that E-cadherin expression level decreased according TGF-β1 stimulation with dose dependent manner; EMT marker, α-SMA, expression level increased and also showed TGF-β1 dose dependent (Figure 2). It was consistency with mRNA level.

Figure 4. Detection of integrin β4 and PPARγ in renal tissue with immunohistochemistry analysis. It showed enhanced expression of integrin β4 in early stage of renal tubular epithelial cell lesions (mild lesion group), but it was over-expression in later stage of renal tubular epithelial cell lesions (moderate lesion group and severe lesion group) comparing mlgG control (Negative control). In contrast, the expression of PPARγ increased lightly in early stage, but it decreased at later stage.
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TGF-β1 increased protein expression level of integrin β4 in HK2 cells

It had been reported that overexpression of integrin β4 promoted cell scattering and cell motility in combination with down-regulation of E-cadherin and up-regulation of Vimentin expression in the regulation of cancer invasion and EMT [19, 20]. High levels of integrin β4 expression correlated significantly with the histological hallmarks.

To validate the association between expression levels of integrin β4 and EMT in TGF-β1 stimulation, immune staining assay was used. We found TGF-β1 stimulation increased integrin β4 expression in HK2 cells (Figure 3). It was shown TGF-β1 dose dependent. HK2 cells had a high degree of solitary cell infiltration with TGF-β1 stimulation.

Integrin β4 over-expression and PPARγ low-expression in the later stage at renal disease

To clarify overexpression of integrin β4 and low expression of PPARγ in renal disease, we examined overall 45 patients with renal diseases. The tubulointerstitial tissues were obtained by biopsy.

Further Histology and immunohistochemistry analysis were performed; integrin β4 and PPARγ were detected in tubulointerstitial tissues (Figure 4). Intriguingly, immunohistochemistry analysis showed enhanced expression of integrin β4 in early stage of renal tubular epithelial cell lesions (mild lesion group), but it was over-expression at later stage of renal tubular epithelial cell lesions (moderate lesion group and severe lesion group). In contrast, the expression of PPARγ was increased lightly in early stage, but it was dramatically decreased at later stage. It is consistency with TGF-β1 inducing EMT.

High levels of integrin β4 expression and low level of PPARγ expression are significantly correlated with EMT by TGF-β1 stimulation.

TGF-β1 signaling pathway involved integrin β4, PPARγ and E-cadherin

Based on the TGF-β1 induced EMT in HK2 cells and the over-expression of integrin β4 and low-expression of PPARγ in tubulointerstitial tissues, we try to understand the relationship of those molecules PPARγ, integrin β4 and E-cadherin.

In our co-immunoprecipitation analysis, HEK-293 cell line was used. PPARγ captured integrin β4. With E-cadherin transfected HEK293 cells, E-cadherin blocked PPARγ capturing integrin β4 (Figure 5). In present E-cadherin, PPARγ dis-associated with integrin β4 and associated with E-cadherin (Figure 5). It was very interesting that the interaction between integrin β4 and PPARγ was interfered by E-cadherin.

Discussion

It is now well accepted that loss of E-cadherin and EMT marker of α-SMA and CTGF expression are key events in EMT. In our previous
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Figure 6. Schematic model of function of TGF-β1 and PPARγ in EMT. Stimulated by TGF-β1, epithelial cells lose cell-cell contacts. E-cadherin. The EMT marker, such as α-SMA etc, was expressed and induced Integrin β4 over-expression. It leads to tubulointerstitial fibrosis. PPARγ inhibits this procedure.

In our study, we found PPARγ were involved in EMT [14].

Integrin β4 is a laminin 332 receptor that forms hemidesmosomes in epithelial cells [26-29]. In tumor cells, overexpression of the integrin promotes the proliferation, survival, and invasion of the tumor cells [30-32]. It is a novel report that integrin β4 also plays a role in the regulation of EMT.

Our immune-precipitation studies showed that integrin β4 associated PPARγ to form a complex but the association can be blocked by adding E-cadherin, suggesting that change of E-cadherin during EMT has effects in functions of PPARγ and integrin β4, however the mechanism is required more investigation. Initiated by TGF-β1, epithelial or endothelial cells lose their cell-cell contacts (loss E-cadherin) and begin to express mesenchymal markers (α-SMA etc.) that undergo EMT, also TGF-β1 induces extracellular matrix expression that contributes to tissue inflammation and cell transdifferentiation [33]. In the interstitium, these cells disengage themselves from the cell connective and transdifferentiate to interstitial myofibroblasts, which are responsible for the increased synthesis of ECM leading to tubulointerstitial fibrosis (Figure 6 modified after [35]). In the glomerulus, the TGF-β1 induced biochemical changes in the cells contribute to excessive ECM deposition and podocyte loss, characteristics of glomerulosclerosis.

Fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses and tissue injury. We have shown that renal diseases related with PPARγ and integrin β4 with our immunohistochemistry assay. In later stages of renal disease, PPARγ expression is decreased while integrin β4 is over-expression.

TGF-β1 thus potentially offers a number of molecular targets for the treatment of renal diseases. Since TGF-β1 is upregulated in almost all progressive renal diseases, TGF-β1-suppressing treatments appear promising [34]. Activation of PPARγ and inhibition integrin β4 has also been considered in the treatment of in chronic kidney disease as a result of renal fibrosis [36]. In addition, several other strategies of TGF-β inhibition have been proven to be effective in the treatment of kidney diseases resulting from tissue fibrosis [37-40].

Acknowledgements

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Disclosure of conflict of interest

None.

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