Intracellular regulation of epithelial-mesenchymal transformation induced by transforming growth factor-β: role of RhoA G-protein and β-catenin

PhD thesis

Dr. András Masszi

Semmelweis University
School of PhD studies

Supervisor: Dr. Rosivall László

Opponents: Dr. Balla József
Dr. Zsembery Ákos

Chairman of commitee: Dr. Reusz György
Members: Dr. Balla József
Dr. Szabó András

Budapest
2006.

Introduction

Progressive, chronic kidney diseases uniformly lead to end stage renal failure. The pathomechanism of these diseases are basically different, however progressive renal fibrosis – as it is identified by the pathophysiological terminology – occurs independently from the original cause. Progressive tissue fibrosis occupies every compartment of the kidney, namely glomerulosclerosis, tubular atrophy, interstitial fibrosis and arteriolosclerosis can be detected.

During tubulointerstitial fibrosis tubular epithelium flattens, brush border disappears, interstitial space widens. Extracellular matrix (ECM) proteins accumulate, and the composition of ECM alters in favour of the fibrillar collagen isoforms. Meanwhile interstitial cells responsible for the production of excess matrix proliferate in the originally cell free environment.

A portion of these cells are myofibroblasts. These contractile cells are characterized by a cytoskeleton component, α-smooth muscle actin (SMA). Experimental and clinical studies showed that myofibroblasts play an important role in the pathomechanism of tubulointerstitial fibrosis. Namely the decrease of the kidney function is proportionate to the number of myofibroblasts in the instersitital space and so does the extent of fibrosis to the expression of SMA.

The origin of these cells in kidney fibrosis however is remained unclear. Discovering epithelial plasticity served as a novel possibility: accordingly epithelial cells in response to transforming growth factor (TGF)-β loose their polarized epithelial morphology and gain a mobile, mesenchymal form. The process characterized by the loss of epithelial cell adhesion, the assembly of stress fibres in the cytoskeleton and the synthesis of mesenchymal markers. Epithelial-mesenchymal transformation (EMT) was detected during both experimental and human kidney fibrosis. Moreover, expression of SMA in tubular cells both in vitro and in vivo clearly proved differentiation to myofibroblast phenotype.

Based on these data a novel pathomechanism of renal fibrosis was suggested: in the initial phase of the disease tubular cells undergo mesenchymal/myofibroblast transformation in response to profibrotic cytokines such as TGF-β and can contribute to the progression.

Members of TGF-β family are polypeptide growth factors, which regulate several cellular functions. The different cellular responses are controlled via a complex intracellular signalling pathway. Although the SMAD protein family is responsible for a variety of TGF-β responses, the existence of alternative pathways were shown. Based on the effects of RhoA, member of the Rho GTP-ase family – stress fibre assembly, reorganization of cell adhesions, SMA expression in mesenchymal cells – we hypothesized that RhoA plays a role in the regulation of EMT induced by TGF-β.

Also the role of cell adhesion components emerged in the regulation of the complex programme of EMT. Namely, β-catenin, component of adherent

junctions acts as a co-activator of T cell factor/Lymphcyte enchancer factor (TCF/LEF) regulate gene expression. It is proven in vitro that the activation of β-catenin/TCF/LEF signalling pathway lead to EMT, further in experimental kidney fibrosis the activation of the pathway was shown. Intriguingly, although the role of β-catenin in TGF-β signalling was suggested, evidence of such mechanisms was not available.

We characterized the phenomenon of epithelial-mesenchymal transformation in tubular epithelial cells, and investigated the intracellular mechanisms employed by TGF-β during the process.

Hypothesises and Aims

Recent findings on epithelial plasticity and epithelial-mesenchymal transformation supported the hypothesis that EMT is implicated in the pathomechanism of progressive renal fibrosis. We investigated EMT induced by TGF-β in proximal tubular epithelial cells. Our purpose was to characterize the phenomenon and clarify the intracellular pathways in the regulation of EMT. We wished to prove the following hypotheses.

1) In proximal tubular epithelial cells the morphological changes induced by TGF-β fulfill the criteria of EMT

We sought an in vitro cellular model, with which EMT can be investigated from several aspects. We wished to investigate several extracellular stimuli on different proximal tubular cell lines. We measured morphological and functional changes.

2) Morphological changes detected during EMT are regulated by the RhoA/ROK pathway.

We planned to measure changes in RhoA GTP-ase activityupon TGF-β treatment. We wanted to show the effect of RhoA by applying constitutive active mutants and to compare this to the effect of TGF-β. We also wanted to show whether the inhibition of Rho would influence the effect of TGF-β. Moreover as a signalling partner of Rho, we investigated the role of ROK by using mutant DNA constructs and chemical inhibitors.

3) RhoA regulates SMA expression in a serum response factor (SRF) dependent manner during EMT induced by TGF-β

We wished to clarify the role of SRF from the Rho-dependent factors. To this purpose we investigated the role of SRF-dependent cis-acting DNA

elements of the SMA promoter. Further we wished to measure the SRF expression during EMT. Finally we aimed to show the activity of SRF upon TFG-β treatment in a functional assay.

4) Cellular responses induced by TGF-β depend on the presence of cell-cell contacts

We wanted to establish alternative models, in which the presence of cell contacts determines whether EMT takes place in response to TGF-β. We wished to compare cellular responses in confluent and non-confluent cultures. Further we planned to examine the effect of TGF-β on a confluent monolayer in a wounding assay or after separating the cells in a calcium free environment.

5) β-catenin, a component of adherent junction participates in the regulation of gene expression during EMT induced by TGF-β

We wanted to detect the intracellular localisation of β-catenin during EMT by both immunochemical microscopy and by biochemical means. Further we wished to show the activation of β-catenin-dependent gene expression by the transfection of an artificial promoter-reporter construct. Also we wanted to investigate cellular metabolism of β-catenin molecules in cells cultured in calcium free medium.

6) β-catenin/TCF/LEF signalling pathway is a prerequisite to SMA expression during EMT induced by TGF-β

We wished to investigate the role of β-catenin/TCF/LEF pathway in SMA expression induced by TGF-β. To this we sought to determine the effect of activation and inhibition of this specific pathway by using SMA promoter plasmids, expression plasmids and pharmacological agents. Furthermore, we wanted to fabricate a retroviral system, to measure SMA protein expression while β-catenin dependent gene expression is inhibited.

Methods

Cell cultures

Experiments were performed with proximal tubular epithelial cells, LLC-PK1 cells. The Cl4 clone selected from the original culture, stably expressing AT1 angiotensin II receptor is the kind gift of Dr. R. Harris.

Transient transfection

Cells were transfected with FuGENE6 reagent. Following serum starvation cells were treated with 10 ng/ml TGF-β for 24 hours. In case of pharmacological inhibitors, cells were pretreated with the agents 30 minutes prior to the TGF-β treatment. Luciferase and β-galactosidase reporter enzyme activities were measured by luminometry. An internal standard plasmid was used to normalize to the errors caused by uneven cell numbers and transfection efficacy.

Western blotting

After treatment cells were lysed in Triton lysis buffer and Triton-soluble and insoluble fractions were separated by centrifugation. Samples were added to Laemmli-buffer and were denatured by boiling. Same amounts of protein were subjected to electrophoresis onto 8-15% reducing polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked in albumin solutions and after vigorous washing were incubated further in primary antibody dilutions. One hour later membranes were washed and incubated with appropriate secondary antibodies conjugated to horse radish peroxidase. Finally bands were visualized by chemiluminescent reaction. Emitted light was captured on film negatives, which were scanned and analyzed further.

Rho-activity assay

Rho activity was determined by performing affinity based precipitation. A chimera protein composed of the Rho-binding domain of Rhotekin a Gluthation-S-transferase was attached to Sepharose beads. After appropriate treatment cells were lysed and activated Rho molecules were captured by adding the fusion protein. The amount of captured RhoA protein was measured by Western blotting.

Myosin light chain (MLC) phosphorylation

The phosphorylation state of MLC was determined by using non-reducing gel electrophoresis. Cells were lysed in acetone containing 10% trichloroacetic acid and dithiotreitol. Precipitates were solved in sample buffer and proteins were separated on 12% urea-glycerol-polyacryl-amide gels. Then proteins were transferred to nitrocellulose membrane and Western blotting was performed by using anti-MLC antibody.

Immunocytochemistry and fluorescent microscopy

Cells were grown on 25 mm coverslips. Following appropriate treatment cells were fixed and blocking in albumin solution. Specimens were incubated with primary antibody followed by secondary antibody conjugated with fluorophore.

Nuclear protein extraction and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were separated according to the description by Deryckere and Gannon. The CArG B sequence (5'-GAGGTCCCTATAGGT-TTGTG-3') was labelled with [32P]ATP, then the probe was incubated with nuclear samples and poly(dA-dT) mix. The samples was subjected to gel electrophoresis. In supershift experiments ant-SRF antibody was added to the samples.

Retroviral infection

To chelate cytoplasmic β-catenin we created a retroviral system. The DNA sequence coding the cyoplasmic domain of N-cadherin and the GFP was cloned into a pFB-Neo vector to create pFB-N-cad plasmid. Similarly pFB-GFP vector was created encoding the GFP gene. Retroviruses were produced in 293T cells and LLC-PK1 cells were infected at 20% confluence. Following treatment cells were lysed and SMA expression was detected by Western blotting.

Statistical analysis

Statistical significance was determined by Student's T-test or analysis of variance and Bonferroni post hoc tests. $p<0.05$ value was accepted to be significant.

Results

1) Proximal tubular epithelial cells undergo epithelial-mesenchymal transformation upon TGF-β treatment.

Characteristic changes of EMT could be detected in LLC-PK1 cells after TGF-β treatment: the loss of epithelial morphology, direct and indirect evidence of increased motility, decreased expression of epithelial markers and increased expression of mesenchymal markers. In addition transformed epithelial cells expressed SMA, which is a characteristic feature of myofibroblast differentiation beyond the mesenchymal phenotype.

2) Rho activation is a prerequisity but not sufficient for EMT induced by TGF-β

RhoA activation was detected in LLC-PK1 cells after short term and long term treatment. Expression of active RhoA mutant caused stress fibre assembly and SMA promoter activation similarly to TGF-β. We applied different constructs to inhibit RhoA. By inhibiting RhoA both cytoskeletal effects and SMA promoter activation could be prevented. However by expressing active RhoA mutant EMT could not be induced as expression of this construct did not lead to SMA protein synthesis.

3) TGF-β activates Rho/SRF pathway, which regulates SMA promoter via its CArG elements.

The expression of SRF is increased due to TGF-β treatment. Further, activation of SMA by mutant Rho was dependent on the presence of CArG B box. Also, in the absence of CArG B box, TGF-β could not activate SMA promoter.

4) The absence of intercellular contacts is a prerequisite however not sufficient to induce EMT

In the presence of matured cell contacts TGF-β could not induce EMT. However disassembly of cell contact (by low confluence, wounding or low extracellular calcium) restores the EMT inducing effect of TGF-β. Distruction of cell contacts alone is not sufficient to initiate of EMT programme. Both disassembly of cells contacts and TGF-β activate SMA promoter. The two stimuli potentiate each others effects.

5) The fate of β-catenin is regulated by the state of cell contacts and the presence of TFG-β

β-catenin is liberated from the cell contacts in low calcium medium. The liberated protein is degradated in the proteosome. However this step is inhibited in the presence of TGF-β. Moreover β-catenin translocates to the nucleus in response to TGF-β and activates TCF/LEF-dependent gene expression.

6) β-catenin, the component of adherent junction acts as a molecular switch facilitates the genetic programme of EMT induced by TGF-β

Elevated free β-catenin – either by co-transfection or by lithium chloride – potentiate the effect of TGF-β on SMA promoter. On the contrary, chelation of free β-catenin inhibits SMA promoter activation and protein expression.

Conclusion

In the experimental models we applied at least two extracellular stimuli were needed to induce the transformation of epithelial cells: namely both the loss of cell-cell contacts and the presence of TGF-β exert their effects. Based on these findings we suggest the “dual injury” model to describe the

pathomechanism of tubulointerstitial fibrosis: Mechanical injury of tubular cells and TGF-β – produced by extrarenal or tubular cells – together induce EMT.

Our results suggest the following molecular mechanism: Activation of Rho by TGF-β leads to cytoskeleton reorganization, increased contractility and SMA synthesis. β-catenin is liberated from the disassembled adherent junctions. Moreover in the presence of TGF-β free β-catenin is rescued from degradation in the proteosome, but promote the genetic programme of EMT

In vivo in chronic kidney disease mechanical injury of the tubular epithelium (shear force, osmotic stress, crystals and mononuclear cells) and autocrine/ paracrine effect of TGF-β together result in tubulointerstitial fibrosis. Presumably, EMT itself can cause TGF-β production and epithelial injury, by generating disassembled cell contacts. This may explain the progressive and chronic feature of kidney fibrosis.

List of publications

Publications on the subject of the thesis presented in peer reviewed journals:

1. **Masszi A**., Di Ciano C., Sirokmány G., Arthur W.T., Rotstein O.D., Wang J., McCulloch C.A.G., Rosivall L., Mucsi I., Kapus A.: Central Role for Rho in TGF-β1-Induced Smooth Muscle Actin Expression During Epithelial-Mesenchymal Transitions. *AJP: Renal Physiology,* 284 (5): F911-24, 2003, IF: 4,344
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